



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>In re</i> Application of:)	
Todd SEELEY)	Group Art Unit: 1636
Serial No. 10/084,700)	Examiner: Sumesh Kaushal
Filed: February 27, 2002)	Atty. Dkt. No. PP-10406.004
For: huBUB3 Gene Involved in Human Cancers		

DECLARATION OF DAVID DUHL

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

I, David Duhl, declare as follows:

1. Before December 1, 1997 I was employed at Chiron Corporation as a Scientist II. although we worked in different research groups, I knew Dr. Todd Seeley and was familiar with his work on the human homolog of the yeast protein BUB3 ("huBUB3"). It was my practice periodically to read and witness Dr. Seeley's laboratory notebooks in which he documented his work with huBUB3.
2. I was asked to identify copies of some laboratory notebook pages which I was told are Exhibits 1-20 attached to a declaration of Dr. Seeley. I was provided with copies of these pages with the dates shown on them. I recognize these exhibits as pages of Dr. Seeley's laboratory notebooks.

3. Each of these laboratory notebook pages contains my signature and the date on which I read and understood the contents of each of these notebook pages. I signed and dated each of these pages after I had read and understood the entries above my signature on each page. These entries on each page were present on the page before I signed it. With the exception of the page identified as Exhibit 20, I signed and dated each one of these pages before December 1, 1997.

4. I declare that all statements I made in this declaration from my own knowledge are true and that I believe all statements I made on information and belief to be true. I made these statements with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2/1/05
Date

David Duhl
David Duhl



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of:

Todd SEELEY

Serial No. 10/084,700

Filed: February 27, 2002

For: **huBUB3 Gene Involved in Human Cancers**

)
) Group Art Unit: 1636
)
) Examiner: Sumesh Kaushal
)
)
) Atty. Dkt. No. PP-10406.004

DECLARATION OF CHUN TING LEE-NG

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

I, Chun Ting Lee-Ng, declare as follows:

1. Before December 1, 1997 and at the time of the events I describe in this declaration, I was employed at Chiron Corporation and I held the position of Specialist 1, working in Chiron's DNA Sequencing Department. I was responsible for carrying out DNA sequencing reactions as requested by Chiron scientists to confirm expected or known sequences or to determine sequences from novel DNAs. All of the events I describe in this declaration took place before December 1, 1997.

2. The sole exhibit that is attached to this declaration contains copies of three pages from my laboratory notebook NB9203. These are true and accurate copies, except that the dates

on which I did or recorded the work I describe on the notebook pages and any information not relevant to this declaration have been removed.

3. At the time of the events I describe in this declaration, my typical practice for determining a DNA sequence was as follows. The scientist would fill out a "Request for DNA Sequence" form and send me the form together with one or more samples of the DNA to be sequenced. The form identified the clone or fragment to be sequenced and the vector containing the clone or fragment. The scientist also would e-mail me a proposed sequence for reference purposes and comparison with sequencing results.

4. Based on the proposed cloned fragment sequence and the sequence of the vector in which the fragment was contained, I would either select appropriate DNA sequencing primers from our primer library (pre-existing set of oligonucleotide primers corresponding to a searchable file containing their sequences) or design and order synthesis of new primers as needed. I used the primers to carry out sequencing reactions using an ABI automated DNA sequencing system. The system typically yielded sequence data from overlapping fragments about 500 nucleotide bases in length. Each overlapping fragment is initiated from a sequence-specific primer annealing site within the submitted DNA sample molecule, and sequence data are generated downstream from the primer's 3' terminus.

5. ABI system DNA sequence text output files from individual sequencing reaction results (a unique primer was used in each reaction) were transferred to a Chiron UNIX-based computer account. The overlapping, individual sequencing results were manually assembled and edited into one contiguous, consensus sequence using the UNIX visual text editor (vi mode). The assembled consensus sequence was then aligned with the scientist's proposed sequence

using the "MALIGN" Unix-based sequence alignment program. I then returned the completed request form to the scientist together with a printed copy of the sequence alignment.

6. Page 1 of Exhibit 1 is a copy of page 23 of my notebook NB9203. At the bottom right of page 23 is a copy of a request form I received from Todd Seeley, which asked me to sequence a clone identified on the request form as "291-2." Page 2 of Exhibit 1 is an enlarged copy of the request form. The request form indicated that clone 291-2 was in a pCR3.1 vector. The request form also indicated that Dr. Seeley provided me with a proposed sequence of 291-2. After the heading "objective" at the bottom of the form, something is crossed out after the words "sequence novel gene." This crossed out entry was on the form when I received it.

7. I documented the primers I selected and the dilution and amount of the DNA preparation I used to sequence 291-2 on page 22 of my notebook NB9203 (items 7-9 towards the bottom of the page) (page 3 of Exhibit 1). I diluted the DNA preparation Dr. Seeley gave me four-fold (1:4) and used 1 λ (1 μ l) of the diluted preparation for each sequencing reaction. As I documented on page 22, I used two vector-derived sequencing primers: the stock DNA sequencing primer "T7," which corresponded to the sense strand of T7 promoter sequences on the pCR3.1 cloning vector, and the stock antisense DNA sequencing primer "JET1521," which was derived from the bovine polyA region of the pCR3.1 vector. Based on Dr. Seeley's proposed sequence I used a primer I identified as "CT303" to use as an internal primer.

8. DNA sequence result test files from the three primer reactions were transferred from the ABI sequencer system to the UNIX-based account, where sequence assembly, editing, and analyses were performed. First, un-clear or un-needed terminal sequencing data were trimmed from each individual result by visually checking against printed copies of ABI system-generated sequence chromatograms (electropherograms) and against vector-insert junction

information provided within the proposed sequence. The antisense JET1521-derived sequencing result was converted to its reverse complement so to read in the same sense direction as the other two primer reaction results. The three overlapping sequences were merged together within a single result text file for the assembly and editing. Overlapping, redundant sequences were visually identified within the terminal sequences of the individual results, and the sequences were joined, after deleting one of the redundant sequence copies, to form the assembled sequence.

9. Page 4 of Exhibit 1 is a copy of page 84 of my notebook NB 9203. Page 84 contains another copy of Dr. Seeley's request form. Page 5 of Exhibit 1 is an enlarged copy of the request form. The bottom of the form indicates that I completed the sequencing of the 291-2 clone and attached a copy of the sequencing results. A copy of the sequencing results is on page 84 to the right of the request form. Page 6 of Exhibit 1 is an enlarged copy of the sequencing results. This is an alignment of Dr. Seeley's proposed sequence of clone 291-2 with the sequence I determined. The sequence I determined matched the proposed sequence Dr. Seeley sent me except at position 632 in the 291-2 sequence on Page 6 of Exhibit 1. The proposed sequence had adenine (A) at this position, whereas the sequence I determined had cytosine (C). According to my typical practice, I would have sent the completed request form and the sequencing results to Dr. Seeley promptly after I completed the sequencing.

10. I declare that all statements I made in this declaration from my own knowledge are true and that I believe all statements I made on information and belief to be true. I made these statements with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such

willful false statements may jeopardize the validity of the application or any patent issuing from the application.

31 MAR 05

Date

A handwritten signature in black ink, consisting of a stylized 'C' followed by a horizontal line and a small flourish.

Chun Ting Lee-Ng

LEE-GN

DECLARATION

EXHIBIT NO. 1

TITLE.

From Page No. _____

REQUEST FOR DATA SEQUENCE

REQUEST FOR DATA SEQUENCING

REPORT FOR DATA SUBMISSION

(All asterisked questions must be completed before service begins)

Question: 1000 Series Ref: 2461 Date:

notebook #: _____ "Project code/name: Exp 12a-h

Requestor agrees that this sequence will not be published or used for any regulatory purposes unless it is later confirmed. (Please initial) _____

[illegible]

271-2 PER21 (see proposed eq.) 1.748/2

Method of DNA preparation: QiaGen

Object: Sequence notation ~~_____~~

Other helpful information: Please attach any information (e.g., construct information and expected sequence) which may facilitate sequence determination
 intent is in sense orientation pcr3,1

received by: _____ Date received: _____

Completed by: _____ Date Completed: _____

Request Number: _____ Notebook No: _____

To Page No. _____

Witnessed & Understood by me,

Date

invented by

Date

Recorded by _____

NB92030

Serial No. 10/084,700

Filed 02/27/2002

LEE-GN DECLARATION

EXHIBIT NO. 1

023

REQUEST FOR DNA SEQUENCING
(All asterisked requester items must be completed before service begins)

requestor: Dodd Sealey Ext. 2461 Date: [REDACTED]

notebook #: _____ Project code/name: EXP 12-6

requestor agrees that this sequence will not be published or used for any regulatory purposes unless it is first confirmed. (Please initial) _____

Insert/Fragment Name	Vector Name	EE sites	Insert Size	Suggested Primers	*DNA Conc (attach photo)
271-2	pCR3.1	(see proposed seq.)			1.742/2

Method of DNA preparation: Qiagen

Purpose: Sequence novel gene ~~(unpublished)~~

Other helpful information: Please attach any information (e.g. construct information and expected sequence) which may facilitate sequence determination
insert is in sense orientation pCR3.1

received by: _____ Date received: _____

Completed by: _____ Date Completed: _____

request number: _____ Notebook ref: _____

Project No. _____

Book No. _____

TITLE _____

From Page No. _____

DMSO	1	[Redacted]	1.5x	CT300	
	2		1.5x	CT305	
	3		1x	CT306	x
	4		1x	CT307	x
	5		1.5x	Jet307	x
	6		1.5x	Jet333	x
	7		1.5x	T7	
	8				
	9				
	10				
	11				
	12				
	13				
	14				

400003

Not enough DNA

DMSO	1	[Redacted]	x	CT306	
	2		↓	CT307	
	3		2.5x	after leader	x
	4		3.0x	↓	x
	5		1.5x	LS907	
	6		1.1x	↓	
	7		1x	T7	
	8		↓	Jet1521	
	9		↓	CT308	
	10		4.5x	GAPP	
	11		2.5x	↓	

291-2 (1:4)

Try DMSO

Wrong primer
Try LS907

Order CT308 for 10/15/12

CT309 for aa238077

To Page No. _____

Witnessed & Understood by me,

[Signature]

Date

[Redacted]

Invented by

Recorded by

[Signature]

Date

[Redacted]

From Page No.____

REQUEST FOR INFO. SEQUENCE
(All asterisked sequencer items must be completed before service begins)
sequencer: Dodd Sealey Ext: 2461 Date: [redacted]
lookbook #: _____ *Project code/name: Exp Tech.
sequencer agrees that this sequence will not be published or used for any
regulatory purposes unless it is 100% confirmed. (Please initial)

Insert/Fragment Name	Vector Name	RE sites	Insert Size	Suggested Primers	*DNA Conc (attach photo)
271-2	pCR3.1	(see proposed seq.)			1.7 ug/2

method of the preparation: Qinigen
objective: Sequence novel gene ~~Sequence novel gene~~

Other helpful information: Please attach any information (e.g. construct information and expected sequence) which may facilitate sequence determination
insert is in sense orientation pcr.1

Received by: _____ Date received: _____

Completed by: Chen Ting Liao Date Completed: _____

Request Number: 765 Notebook Ref: _____

Final Sequence Computer Pathway: 45-

Printed by: (For novel sequences)

COMMENTS: Science copy attached.

SOURCE NAMES, THEIR LENGTHS and ALIGNMENTS

# 1:	hybub3.ref	1068	99.814
# 2:	291-3	1000	97.899

In alphabet in which alignment was found.

[illegible]

To Page No. _____

Witnessed & Understood by me,

Date**Invented by****Date**

Recorded by _____

REQUEST FOR DNA SEQUENCE

(All asterisked requester items must be completed before service begins)

Requestor: Todd Sealey Est. 2461 Date: [redacted]
 Notebook #: _____ Project code/name: Exp Tech.

Requestor agrees that this sequence will not be published or used for any regulatory purposes unless it is 100% confirmed. (Please initial) _____

Insert/Fragment Name	Vector Name	RR sites	Insert Size	Suggested Primers	*DNA Conc (attach photo)
271-2	PCR3.1	(see proposed seq.)			1.742/2

Method of DNA preparation: Qiagen
 Objective: Sequence novel gene ~~XXXXXXXXXXXX~~

Other helpful information: Please attach any information (e.g. construct information and expected sequence) which may facilitate sequence determination
insert is in sense orientation PCR3.1

Received by: _____ Date received: _____
 Completed by: Chun Ting Lee Date Completed: [redacted]
 Request Number: 7665 Notebook Ref: _____
 Final Sequence Computer Pathway: _____
 Proof-read by: (for novel sequences) _____
 Comments: Sequence seqs attached.

# 1:	hjbnp2.ref	1058	99.81%
# 2:	291-2	1000	97.89%

0 {GGGAGCCCAAGATTCACCGCTTCTAACAGATTTCAGCTGAACCAACCA
0 ymptecgggtt {GGGAGCCCAAGATTCACCGCTTCTAACAGATTTCAGCTGAACCAACCA

47 CCGAGCATGUCATCTCCTCCGTGAAGTTCAAGCCCCAACAACCTCCCASTTCTTGCTGTG
58 CCGAGCATGUCATCTCCTCCGTGAAGTTCAAGCCCCAACAACCTCCCASTTCTTGCTGTG

107 TCTCTCTGGGCACAGTCCGTGCGTCTGTACAGATGTGCCGCAACTCCAATGGGCTCAAG
118 TCTCTCTGGGCACAGTCCGTGCGTCTGTACAGATGTGCCGCAACTCCAATGGGCTCAAG

167 TACCACACACCGGCGCGGTCCGTGCACTGCCGCTTCTACAGATCCAAACGCATGCCCTGGAG
178 TACCACACACCGGCGCGGTCCGTGCACTGCCGCTTCTACAGATCCAAACGCATGCCCTGGAG

227 GGAGGACTAGATCATCAATTGAAAATGCATGATTTGUAACACTGATCAAGAAAATCTGTT
238 GGAGGACTAGATCATCAATTGAAAATGCATGATTTGUAACACTGATCAAGAAAATCTGTT

287 GGGACCCATGATGCCCTATACAGATGTGTTGAATACTGTCCAGAAGTGAATGTGATGGTC
298 GGGACCCATGATGCCCTATACAGATGTGTTGAATACTGTCCAGAAGTGAATGTGATGGTC

347 ACTGGAAGTTGGGATCAGACAGTTAAACTGTGGGTCCCAACTGCTTGTATGCTGCGG
358 ACTGGAAGTTGGGATCAGACAGTTAAACTGTGGGTCCCAACTGCTTGTATGCTGCGG

407 ACCTTCTCTCACGCTGAAGAGGTATATACCTCTCAAGTGTCTGAGAGCGGCTGATTTGTG
418 ACCTTCTCTCACGCTGAAGAGGTATATACCTCTCAAGTGTCTGAGAGCGGCTGATTTGTG

467 GGAACACGACGCGCGAGAGTGTGTGTGTGGGACTTAAGGACATGTGTTACCTGCAAGCAG
478 GGAACACGACGCGCGAGAGTGTGTGTGTGGGACTTAAGGACATGTGTTACCTGCAAGCAG

527 CGCAGGAGTCCAGGCTGAAATACCAACTGCTGTCATACAGAGGCTTCCAAACAGAGCAG
538 CGCAGGAGTCCAGGCTGAAATACCAACTGCTGTCATACAGAGGCTTCCAAACAGAGCAG

587 GGTATGTTATTAGCTCTATTEMAGGCGAGTGG} a [AGTTKSTATTTBAGCCCANB
598 GGTATGTTATTAGCTCTATTEMAGGCGAGTGG} c [AGTTKSTATTTBAGCCCANB

643 CCTGAGGTACAGAGAGAGAGTATGCCCTTCAAATGTCAAGACTAAAGAGAAATATAT
654 CCTGAGGTACAGAGAGAGAGTATGCCCTTCAAATGTCAAGACTAAAGAGAAATATAT

703 TGAGCAGATTACTCACTCATGCCATTTCCTTTCAGATATCCCAATACATTTGCCAC
714 TGAGCAGATTACTCACTCATGCCATTTCCTTTCAGATATCCCAATACATTTGCCAC

763 AGGTGCTTCTGATGCGCTTTGTAAATATTGGGATGCCATTTAACAGAAAGOGACTGTGCCA
774 AGGTGCTTCTGATGCGCTTTGTAAATATTGGGATGCCATTTAACAGAAAGOGACTGTGCCA

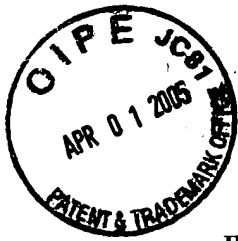
823 ATTGCATGGGTACCCAGGAGCTCCATGACTTGCCTTCAATATGATGGGACTACGCT
834 ATTGCATGGGTACCCAGGAGCTCCATGACTTGCCTTCAATATGATGGGACTACGCT

883 TGCANTAGGCTCATATATATGATGAAATGATGACACAGACATGCTGAGATGATAT
894 TGCANTAGGCTCATATATATGATGAAATGATGACACAGACATGCTGAGATGATAT

943 CTTCCTTCGCCAGTGAACAGATGACAAACAAACCCCAATGCACCATGTACTTGACAGAG
954 CTTCCTTCGCCAGTGAACAGATGACAAACAAACCCCAATGCACCATGTACTTGACAGAG

1083 TTTCATTACTTAGTGCCTTTTGTGTAATTAANCAATTCCTACTGCCCAATGCTGG
1014 TTTCATTACTTAGTGCCTTTTGTGTAATTAANCAATTCCTACTGCCCAATGCTGG

1053 ATTT) 1.....
1074 ATTT) agccgagatc



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
Todd SEELEY) Group Art Unit: 1636
Serial No. 10/084,700) Examiner: Sumesh Kaushal
Filed: February 27, 2002) Atty. Dkt. No. PP-10406.004

For: **huBUB3 Gene Involved in Human Cancers**

DECLARATION OF LING WANG

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

I, Ling Wang, declare as follows:

1. Before December 1, 1997 I was employed at Chiron Corporation. At the time of the events I describe in this declaration, I held the position of Research Specialist I. I was involved in projects to identify novel human genes and analyze their functions.

2. Before December 1, 1997, I participated in work leading to the cloning and sequencing of the open reading frame of the human BUB3 (huBUB3) gene. I describe some of my work on the huBUB3 project in the paragraphs that follow. All of this work was carried out in the United States before December 1, 1997. In addition, all of the events identified below also occurred before December 1, 1997.

3. The exhibit that is attached to my declaration contains true and accurate copies of pages from my laboratory notebook 9210 except that the dates on which I did the work I describe on the notebook pages have been removed. There are two copies of each of the pages; these two copies were electronically scanned so that all parts of the page's contents can easily be seen.

4. Dr. Seeley provided me with plasmids containing cDNA inserts so that I could screen the plasmids for him and identify those with cDNA inserts of the correct size and orientation. I noted at the top of page 17 of my notebook no. 9210 that Dr. Seeley did the ligations and PCR reactions to make these plasmids. Exhibit 1, pages 1 and 2. I knew that the purpose of the ligations was to insert a huBUB3 cDNA into the plasmids. I knew that the size of the huBUB3 cDNA insert was about 1 kb.

5. I did this screening by purifying miniprep DNA from the plasmids, digesting the DNA with various restriction enzymes, and separating the fragments by gel electrophoresis. Before I did the digestions I had a copy of the huBUB3 cDNA nucleotide sequence provided to me by Dr. Seeley. Dr. Seeley and I discussed the sequence when we chose which restriction enzymes to use.

6. I digested samples of each plasmid miniprep DNA with the restriction enzyme *EcoRI* to verify that the insert sizes corresponded to the size of the PCR product. I digested other samples of miniprep DNA with *Asp718* and *NotI* to verify the orientation of the insert, based on the expected location of these sites within the predicted sequence of the insert. I describe these restriction enzyme digestions on pages 17-18 of my notebook no. 9210; Exhibit 1, pages 1-4).

7. From these digestions, I determined that plasmids from clone 2 (clone "291-2") contained a huBUB3 cDNA insert of the correct size and in the correct (sense) orientation. As I

indicated at the bottom of page 18 of my notebook (Exhibit 1, pages 3 and 4), my next step was to make a large scale preparation of this clone and send it to Chiron's FastTrack DNA sequencing service for sequencing.

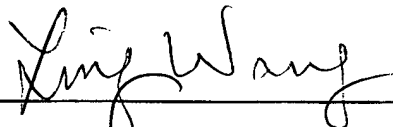
8. I ordered and/or prepared all the reagents I used for these experiments.

9. I attended many lab meetings with Dr. Seeley at which huBUB3 was discussed. I believe that Dr. Michael A. Innis and Elizabeth Scott were at some of these meetings. I also remember that Dr. Seeley presented the huBUB3 work to staff of the Research Department at a "Chiron Friday Tech-Talk."

10. Dr. Seeley and I regularly discussed our experimental results. I remember seeing the Northern blots that demonstrated differential expression of huBUB3 in different human tissues.

11. I declare that all statements I made in this declaration from my own knowledge are true and that I believe all statements I made on information and belief to be true. I made these statements with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/24/05
Date


Ling Wang

WANG

DECLARATION

EXHIBIT NO. 1

TITLE

hu BUD3 analysis

Project No. _____

Book No. _____

17

From Page No. _____

289 5' RACE

290 5' RACE

291 hu BUD3

To do did PCR and ligation

289 — 290 — 291 — vector control

Digest with EckRI.

289 — 2

290 — 8 digest with NotI

Result all are vectors.

Digest with Asp718, all are sense orientation

Pick up more 291 clones.

Witnessed & Understood by me,

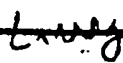


Date



Invented by

Recorded by



Date



To Page No. _____

NB92100

Serial No. 10/084,700

Filed 02/27/2002

WANG DECLARATION

EXHIBIT NO. 1

017

TITLE

lin B11D3 analysis

Project No.

Book No.

17

From Page No.

289 3' RACE

290 5' RACE

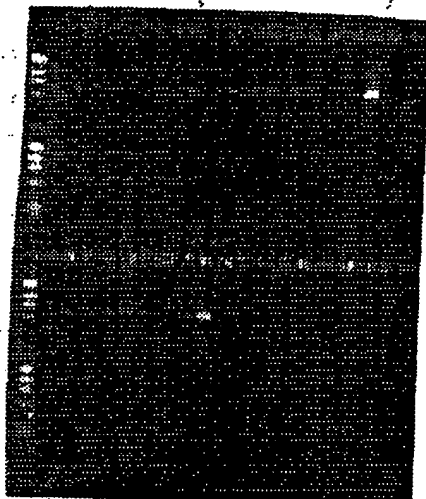
291 lin B11D3

Totd did PCR and ligation

289 — 8 — 290 — 8

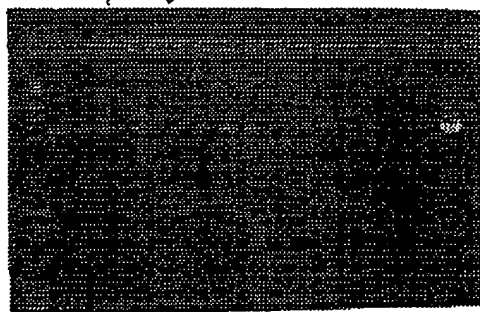
vector control

Digest with EcoRI



289 — 8

290 — 8 Digest with EcoRI



Results: all are vectors

291

1 — 8

291.2
291.3
291.4
291.5
291.6
291.7

Digest with Acp718, all are same restriction

Pick up more 291 clones



To Page No.

Witnessed & Understood by me.

Date

Invented by

Date

X

Recorded by

Living

NB92100

017a

Project No. _____

Book No. _____

TITLE

BUB-3 analysis Result.

From Page No. _____

291-9
291-13
291-16
291-18
291-24
291-30
vector

291-9 → 291-30 chg by

Asp 718

~~291-23~~
ply of 291-13, 291-16, 291-18

291-21, 291-26, 291-27

291-28, 291-30 with Not 1

To conform the orientation
of the insert.

No.	A1	A2	A1-A2	A1/A2
1	0.000	-0.004	0.004	0.0000
2	0.211	0.110	0.101	1.9222

2.11-8/λ

— Large scale prep. 291-2 and send to FASTrack sequence.

1 -0.000 0.005 -0.005 -0.0230

2 0.100 0.105 0.093 1.7854 291-2

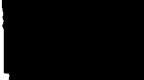
1.88-8/λ

To Page No. _____

Witnessed & Understood by me,



Date



Invented by

Recorded by

L. Wang

Date



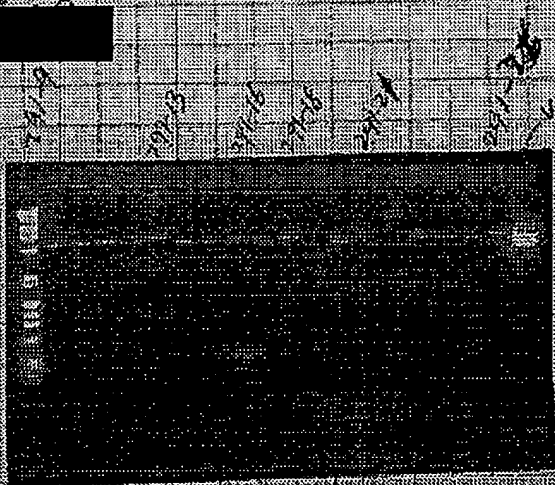
Project No. _____

Book No. _____

TITLE

BUB-3 summary Remit.

From Page No. _____



291-9 → 291-30 closed by

Asp 218

291-13, 291-16, 291-18

291-21, 291-26, 291-27

291-28, 291-30 with Not 1

To conform the unit
of the insert.

No. A1 A2 A1-A2 A1/A2

1 0.000 -0.004 0.004 0.0000

2 0.211 0.110 0.101 1.9222 EGPA

2.1128/x

Large scale map 291-2 and send to FASTrack sequence

1 -0.000 0.005 -0.005 -0.0238

2 0.130 0.105 0.083 1.7854 291-2

1.5328/x

To Page No. _____

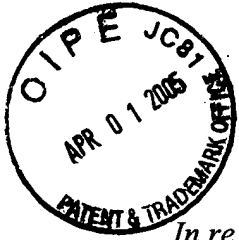
Witnessed & Understood by me,

Date

Invented by

Date

Recorded by



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Todd SEELEY

Serial No. 10/084,700

Filed: February 27, 2002

)
) Group Art Unit: 1636
)
) Examiner: Sumesh Kaushal
)
)
) Atty. Dkt. No. PP-10406.004

For: **huBUB3 Gene Involved in Human Cancers**

DECLARATION OF TODD SEELEY

U.S. Patent and Trademark Office
Customer Service Window
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

I, Todd Seeley, declare as follows:

1. Before December 1, 1997 I was employed at Chiron Corporation. At the time of the events I describe in this declaration, I held the position of Scientist II. I was responsible for research in the Cancer Targets and Mechanisms group under the direction of Lewis T. (Rusty) Williams, President, Chiron Technologies.

2. Before December 1, 1997, I and those under my direction cloned and sequenced the open reading frame of the human BUB3 ("huBUB3") gene, expressed the huBUB3 protein from the cloned sequence, and demonstrated the protein's function. I describe these experiments and some related events in the paragraphs that follow. All of these experiments were carried out in the

United States before December 1, 1997. All of the events I describe took place in the U.S. before December 1, 1997.

3. Each of the exhibits I refer to in the following paragraphs is a true and accurate copy of the original except that descriptions of material not relevant to huBUB3 and the dates on which the work I describe was done and recorded have been removed. There are two copies of some of my notebook pages (*i.e.*, “page 88-88a”); these two copies were electronically scanned so that all parts of each page’s contents can be seen.

4. Before I began the work I describe in the paragraphs below, the yeast proteins BUB3 and BUB1 were known to be involved in the normal control of mitosis in yeast. A complex of both proteins is required for cell cycle arrest to occur in response to disruption of the spindle by microtubule poisons. Hoyt *et al.*, *Cell* 66, 507-17, 1991 (Exhibit 21); Roberts *et al.*, *Mol. Cell Biol.* 14, 8282-91, 1994 (Exhibit 22). I was interested in identifying human proteins which carry out similar functions, because microtubule poisons, such as vinblastine, vincristine, and taxol, are commonly used cancer chemotherapeutic agents. I thought that proteins which would interfere with cell cycle arrest in cancer cells treated with microtubule poisons would be useful therapeutic targets. I reasoned that, if cell cycle arrest did not occur, the effect of the microtubule poison could be potentiated.

5. For this reason, I believed it would be useful to search for human homologs of the yeast proteins BUB1 and BUB3. Before December 1, 1997, I identified a human homolog of BUB1 (huBUB1), which I describe in U.S. Patent 6,489,137 (Exhibit 23).

6. I documented my strategy for identifying a human homolog of BUB3 on page 78 of my lab notebook no. 8947 (“NB-8947”) (Exhibit 1, page 1). First, I “reverse translated” the *S. cerevisiae* BUB3 protein sequence to obtain nucleotide sequences which could encode the yeast

protein. I used these sequences to conduct searches of public databases using Chiron's "MASPAR" (massively parallel) computer and the program MPSrch. My searches identified a mouse cDNA clone (GenBank Accession No. U67327), which was annotated in GenBank as a "WD40-repeat type I transmembrane protein A72.5." See paragraph 1 of my invention disclosure (Exhibit 2; described below). Page 78 of my notebook shows an alignment between the predicted amino acid sequence encoded by U67327 and the amino acid sequence of yeast BUB3 (Exhibit 1, page 1). The similarities between the two proteins indicated to me that U67327 was a potential murine homolog of the *S. cerevisiae* gene scBUB3. See page 78 of my notebook.

7. I then used the nucleotide sequence of U67327 as well as the nucleotide sequences generated by reverse translating the yeast BUB3 amino acid sequence to identify human expressed sequence tags (ESTs) which spanned the entire U67327 nucleotide sequence. I compiled these EST sequences into a single coding sequence of what I identified as a human BUB3 homolog ("huBUB3"). This sequence is shown in the middle of page 79 of NB-8947 (Exhibit 1, page 2).

8. I wrote an invention disclosure describing the huBUB3 nucleotide sequence and I put a copy of the disclosure into my notebook. Exhibit 2, pages 82-85 of NB-8947. The compiled huBUB3 nucleotide sequence spans pages 82 and 83. My invention disclosure also contains the predicted amino acid sequence of the huBUB3 protein encoded by the huBUB3 nucleotide sequence (page 84) and describes how huBUB3 would have useful applications in situations in which microtubule poisons are used, such as in cancer chemotherapy (pages 84-85). I also explained how the huBUB3 protein could be useful in cancer diagnostics and in the development of new therapeutics (page 85).

9. I sent my invention disclosure to Chiron's Intellectual Property Department by inter-office mail, (see my notation at the top of page 82). Ling-Fong Chung of Chiron's

Intellectual Property Department assigned the reference no. E-90-0008 to my invention disclosure, which I noted on page 91 of NB-8947 (last page of Exhibit 2).

10. Based on the compiled huBUB3 nucleotide sequence, I designed a primer pair (sense primer TWS95, 5'-GGGAGCCCAAGATGACCGGTT; antisense primer TWS96, 5'-AAATCCACCATTTGGGGAGTACGAATTGT) that I could use to amplify huBUB3 cDNA from a cDNA library. These primers are described at the bottom of page 79 of NB-8947 (Exhibit 1, page 2).

11. I planned to use the primer pair TWS95 and TWS96 to amplify a huBUB3 cDNA clone from a human testis cDNA library using polymerase chain reaction (PCR). The sequences of the two primers are shown again on the left side of page 87 of NB-8947 (Exhibit 3, page 1). I expected a huBUB3 cDNA clone to have a size of approximately 1 kb; I documented my expectation on the right-hand side of page 87. I chose testis as an initial source of huBUB3 RNA because it expresses many genes poorly detected elsewhere.

12. I described the PCR reaction conditions on page 88-88a of NB-8947 (Exhibit 3, pages 2 and 3). This PCR reaction was intended to amplify the coding region only (there are untranslated regions at the 3' and 5' ends that were not amplified by this PCR reaction). On the bottom of page 88-88a there is a photograph of an ethidium bromide-stained minigel that shows the size of the amplified cDNA product. The band corresponding to the amplified cDNA is in lane 4; this band has a size of approximately 1 Kb, which is consistent with the size I expected for a full-length ORF huBUB3 cDNA.

13. I ligated the huBUB3 cDNA into a pCRTM3.1 plasmid vector (this was ligation preparation no. 291, as indicated at the bottom of page 93 of NB-8947; Exhibit 4, page 1); a diagram of the vector is shown on page 94-94a of NB-8947 (Exhibit 4, pages 2 and 3). I inoculated

eight 3 ml cultures of Luria-Bertani (LB) broth with clones of ligation preparation no. 291, as I described at the top of page 93 of NB-8947 (Exhibit 4). As I indicated on the bottom of page 93 of NB-8947, I gave the cultures to my technician, Ling Wang, to purify miniprep DNA and screen for the size and orientation of the cDNA inserts.

14. Ms. Wang digested plasmids from each of these minipreps with restriction enzymes and separated the fragments by gel electrophoresis. From these digestions, Ms. Wang determined that plasmids from clone 2 (clone "291-2") contained a cDNA insert of the correct size and in the correct (sense) orientation. Ms. Wang used an *EcoRI* to verify that the insert size corresponded to the size of the PCR product. She used *Asp718* and *NotI* restriction digests to verify the orientation of the insert, based on the expected location of these sites within the predicted sequence of the insert (pages 17-17a of Ms. Wang's notebook no. 9210; Exhibit 5, pages 1 and 2). As she indicated at the bottom of page 18-18a (Exhibit 5, pages 3 and 4), Ms. Wang planned to make a large scale preparation of this clone and send the preparation to Chiron's FastTrack DNA sequencing service.

15. I noted the results of the FastTrack DNA sequencing on page 114 of NB-8947 (Exhibit 6). Two vector derived sequencing primers were used. A stock DNA sequencing primer ("T7", corresponding to the sense strand of T7 promoter sequences on the cloning vector) was used to read sequence from the PCR product, including the cloned TWS95 primer and thereafter into the cloned huBUB3 cDNA sequences adjacent to the TWS95 primer sequence. Similarly, a stock antisense DNA sequencing primer ("JET1521," derived from the bovine polyA region of the vector) was used to read cloned huBUB3 sequences from the opposite TWS96 end of the cloned insert. From the notations on this page, it is apparent that this preliminary sequence data left a small gap of 47 nt corresponding to the central-most region of the insert (nucleotides 333-380).

16. I also identified other huBUB3 cDNA clones from ligation 291. Page 135-135a of NB-8947 (Exhibit 7, pages 1 and 2) documents the identification of 4 additional clones of huBUB3 cDNA-containing plasmids. I made mini-preparations of these clones and confirmed that plasmids in each clone had a huBUB3 cDNA insert using an *EcoR*I digestion. As I indicated at the bottom of page 135-135a, I grew a larger preparation of one of these clones (clone no. 291-45) with an antisense orientation, defined by the appearance of an Asp718 restriction digest fragment of ~200 nt. As I indicated on page 136-136a of NB-8947 (Exhibit 7, pages 3 and 4), I wanted to have a huBUB3 probe to use for Northern blots. I digested plasmids from the new preparation of 291-45 with *EcoR*I to obtain a huBUB3 cDNA fragment I could use to prepare a probe.

17. The synthesis of a ³²P-labeled probe derived from the clone 291-45 *EcoR*I cDNA fragment is shown on page 157 of NB-8947; a copy of the X-ray film showing the hybridization signals is on page 157a-c (Exhibit 8). I hybridized this probe to a commercial Northern blot containing mRNA isolated from a variety of human organs; the protocol I used is described on page 138 of NB-8947 (Exhibit 8, page 5). The probe hybridized to an mRNA of the expected size (~1.4 kb), along with additional species. This mRNA is expressed at low levels in tissues from spleen, thymus, prostate, ovary, small intestine, colon mucosa, and peripheral blood leukocytes. This mRNA is significantly over-expressed in adult testis, which is a tissue with a high level of cell proliferation. These results indicated to me that the expression of huBUB3 is widely distributed, and suggested that huBUB3 performs a common function in cell proliferation shared by cells in various tissues.

18. I summarized my work on huBUB3 for a patent meeting. The summary is on page 159 of NB-8947 (Exhibit 9). I believe Ling-Fong Chung was present at the meeting.

19. Chun Ting Lee-Ng, who was a member of the DNA sequencing core facility staff at Chiron, sequenced the entire huBUB3 sense clone no. 291-2. The sequence of the cloned cDNA matched the predicted sequence assembled from the human ESTs except for a cytosine (C) at position 667, where I had selected adenine (A) in the compiled sequence. Some of the ESTs I used to compile the sequence had a C in this position, some had "N" (unknown), but more of them had an A. I decided that the compiled sequence should have A at this position. The alignment of the sequenced clone and the predicted nucleotide sequence is shown on pages 175 and 176 of NB-8947 (Exhibit 10).

20. I presented my work on huBUB3 at another patent meeting and recorded this information on pages 30, 31, and 33-34a of NB-8948 (Exhibit 11). My presentation included a discussion of yeast spindle defect signaling (page 31), comparisons between mouse and human BUB3 amino acid sequences (page 33-33a-33b), and the over-expression of huBUB3 in testis (page 34-34a). I prepared an addendum to my first huBUB3 invention disclosure, which I gave to my contact in Chiron's Intellectual Property Department, Ling-Fong Chung. I put a copy of the addendum on pages 42-44b of NB-8948 (Exhibit 12).

21. Around the same time I discussed the over-expression of huBUB3 in testis at a lab meeting with Ling Wang and Beth Scott. Copies of huBUB3 data that I presented in the lab meeting, including a Northern blot showing this over-expression, are on page 78-78a of NB-8948 (Exhibit 13).

22. We engineered a huBUB3-FLAG fusion expression construct (p322-1) by inserting a synthetic FLAG immuno-peptide coding sequence between the *Pst*AI and *Hind*III sites of huBUB3 clone p291-2. This construct contained a synthetic N-terminal methionine codon followed by a sequence coding for three FLAG repeats separated by glycine codons, which is

followed by 5 glycine codons. The design of this sequence is described on page 87-87a of NB-8948 (Exhibit 14, pages 1 and 2). My notes on this page indicate that I designed the fragment with *HindIII* and *AgeI* compatible ends.

23. This segment was synthesized as two long hybridizing oligonucleotides (TWS134 and TWS135). These oligonucleotides were then ligated into polymers and re-digested, to ensure that the resulting purified FLAG fragment would have phosphorylated ends compatible with high-efficiency ligation. This is described on page 95 of NB-8948 (Exhibit 14, page 3). We fused this segment in-frame upstream of the native huBUB3 ATG codon in the huBUB3 reading frame.

24. We digested the 291-2 plasmid with *PinA1* and *HindIII* described on page 94 of NB-8948 (Exhibit 14, page 4). *PinA1* is an isoschizomer of *AgeI* and cuts the same sequence and generates the same ends as *AgeI*. This fragment and the FLAG leader were then ligated to insert the FLAG leader immediately upstream from the huBUB3 start codon. The ligation is described on page 133 of NB-8948 (Exhibit 14, page 5), with a diagram showing the replacement of an upstream segment with the ~90 nucleotide FLAG sequence. The diagram indicates a method used for identifying positive clones from this ligation; its use is described on page 138-138a of NB-8948 (Exhibit 14, pages 6 and 7).

25. On page 149-149a of NB-8948 (Exhibit 14, pages 8 and 9), I describe the large scale preparation of DNA from the positive clone designated p322-1. The plasmid vector we used contained a bacteriophage T7 binding site, which permits *in vitro* synthesis of the encoded transcript.

26. I used a TNT® reticulocyte lysate system and a T7 RNAP-based transcription/translation kit (Promega). I noted this on page 62 of NB-10148 (Exhibit 15, page 1). I carried out the *in vitro* transcription/translation using various plasmids, including plasmid 322-1

(which encoded FLAG-huBUB3) and plasmids 291-2 and 291-45 (which encoded huBUB3) using the reaction conditions and reagents I described on pages 62 and 69 of NB-10148 (Exhibit 15, pages 1 and 2). This reaction produced ^{35}S -methionine-labeled proteins, which I separated by SDS-PAGE using a 10-20% gel, as I describe on the bottom of page 69. I then fixed the gel, dried it, and exposed it to X-ray film to visualize the ^{35}S -labeled proteins.

27. The results of the *in vitro* transcription/translation reaction are shown on pages 71-72a of NB-10148 (Exhibit 16). I calculated molecular weights of the ^{35}S -methionine-labeled proteins based on their relative mobility on the SDS-PAGE gel. I wrote the calculated molecular weights of huBUB3 and FLAG-huBUB3 at the bottom right of page 71-71a (Exhibit 16, page 1). I put the envelope in which I stored the exposed X-ray film on page 72 of NB-10148 (Exhibit 16, page 2). Page 72a is a copy of the X-ray film itself (Exhibit 16, page 3). Constructs with a sense orientation of the insert produced labeled bands which corresponded to huBUB3 and FLAG-huBUB3. There were no bands produced with vector only or with the antisense huBUB3 plasmid 291-45. This result indicated to me that the "input" DNA used in the *in vitro* transcription/translation reaction produced labeled protein products as I expected.

28. I repeated the *in vitro* transcription/translation reaction and used the proteins it produced to demonstrate that an anti-FLAG monoclonal antibody could immunoprecipitate the FLAG-huBUB3 fusion protein. I carried out co-immunoprecipitation assays of the huBUB3-FLAG fusion product using an anti-FLAG monoclonal antibody conjugated to agarose ("α Flag agarose"). I separated fractions of supernatant and agarose bead pellet fractions by SDS-PAGE (I indicated this at the upper right of page 101 of NB-10148; Exhibit 17, page 1). In this experiment, various amounts of input DNA were used to identify optimum conditions for observing FLAG immunoprecipitation.

29. Following incubation at 30 °C for about 1.5 hr, I prepared aliquots of *in vitro* translated protein and added agarose beads conjugated with anti-FLAG monoclonal antibody that had been washed and suspended in TNE buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 2 mM EDTA). After 45 minutes at room temperature, I collected antibody beads by brief centrifugation. I washed the beads twice with TNE before resuspending them in SDS-PAGE sample buffer.

30. I then analyzed aliquots of both the supernatant and the washed pellet fractions by SDS-PAGE on 10-20% gradient gels. I dried the gels and exposed them to X-ray film to visualize the ³⁵S-labeled proteins. I put the envelope in which I stored the exposed X-ray film on page 102 of NB-10148 (Exhibit 17, page 2). Page 102a is a copy of the X-ray film itself (Exhibit 17, page 3). I noted that the intensity of the FLAG-huBUB3 band (the upper band) increased with larger amounts of antibody beads, but that the huBUB3 band (the lower band) did not. This demonstrated to me that the anti-FLAG monoclonal antibody bound to the FLAG epitope of the FLAG-huBUB3 fusion protein.

31. Because binding of the yeast BUB3 protein to yeast BUB1 is required for cell cycle arrest to occur, I wanted to test whether the translated huBUB3 protein could bind to the human homolog of BUB1 (huBUB1). I carried out another *in vitro* transcription/translation reaction with a mixture of plasmids, including plasmids encoding huBUB3, FLAG-huBUB3, and huBUB1. I described this reaction on page 109 of NB-10148 (Exhibit 18, page 1). I modified the immunoprecipitation protocol to include 1% NP-40 in the wash buffer, and documented the revised protocol on page 109.

32. I separated the translated proteins by SDS-PAGE using a 10-20% SDS-polyacrylamide gel. I then dried the gel and exposed it to X-ray film to visualize the ³⁵S-labeled proteins. I put the envelope in which I stored the exposed X-ray film on page 110 of

NB-10148 (Exhibit 18, page 2). Pages 110a and 110b are copies of the X-ray film itself (Exhibit 18, pages 3 and 4). The upper autoradiogram has the singly translated protein reactions, which could be used as a guide to label the proteins. The huBUB3 and FLAG-huBUB3 bands appear in the center portions of each gel, migrating roughly half the way down the gel. The huBUB3 is the faster migrating band, slightly nearer the bottom of the gel, and the FLAG-huBUB3 is the upper band in the mid-portion of the gels. The smear near the top of the gel is huBUB1.

33. I described the results of this experiment on page 111 of NB-10148 (Exhibit 18, page 5). I concluded from this study that FLAG immunoprecipitation could be used to observe association between BUB1 and FLAG-BUB3.

34. I repeated the co-immunoprecipitation experiment with FLAG-huBUB3 and huBUB1; I describe the reaction conditions and results on pages 151-152 of NB-10148 (Exhibit 19, pages 1 and 2). I put the envelope in which I stored the exposed X-ray film on page 153 of NB-10148 (Exhibit 19, page 3). Page 153a is a copy of the X-ray film itself (Exhibit 19, page 4). Lanes 7 and 9 have new bands corresponding to immunoprecipitated huFLAG-BUB3 and huBUB1. In lane 5, huBUB1 could not be immunoprecipitated.

35. These experiments demonstrated to me that huBUB1 was co-precipitated with FLAG-huBUB3 (lane 7), which indicates that huBUB3 is a ligand for huBUB1. When plasmid encoding huBUB3 was added to a subsequent reaction containing FLAG-huBUB3 and huBUB1 plasmids, only labeled FLAG-huBUB3 and huBUB1 were enriched in the pellet (see lane 9 on p102a; Exhibit 17, page 3). These results are consistent with a model in which huBUB3/huBUB1 complexes retain a single monomer of huBUB3 per complex. In this manner, the labeled FLAG-huBUB3 protein competes with huBUB3 for a single binding site in the complex, such that

prior binding of labeled FLAG-huBUB3 precludes association with huBUB3 protein. These results are also consistent with a model in which huBUB3 does not self associate.

36. When I knew that huBUB3 bound to huBUB1, it confirmed my belief that these proteins are useful for identifying agents for disrupting huBUB3-huBUB1 binding and, therefore, for potentiating the effect of microtubule poisons used to treat cancer.

37. I prepared a list of recent results for a meeting with Rust Williams at Chiron. The list is on page 84 of NB-10149 (Exhibit 20). The list refers to the production of huBUB3 antibodies (see item 4 at the bottom of the page). These antibodies were raised by direct DNA immunization of mice with a huBUB3 expression construct (plasmid 191-2) (see paragraph 13, above).

38. I declare that all statements I made in this declaration from my own knowledge are true and that I believe all statements I made on information and belief to be true. I made these statements with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

03 Mar 05
Date

Todd Seeley Ph.D.
Todd Seeley, Ph.D.

SEELEY
DECLARATION
EXHIBIT NO. 1

From Page No.

MASPAR searches with
 Reverse-translated BUB3 yields many hits;
 and one full length m. cDNA: MAUG327,
 whose annotations suggest this is some sort of
 reverse-genetics clone - "transmembrane protein
 A72.5". ~~Just~~ BUB3 binds to BUB1 kinase and
 MAD3 protein.

Session Name:chiremv 2

1. BUB3 (1-341)

PD40

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Initial Score      =    62  Optimized Score =    148  Significance =    0.71
Residue Identity  =    28%  Matches          =    75  Mismatches   =    133
Gaps              =    28  Conservative Substitutions =    28
```

30 40 50 60 70 80 X 90
 RSLILLITSWDGSGLTVYKFDIQAKNVDLLQSLRYKHPILLCCNFIDNTDLQIYVGTVQGEILKVDLLIGSPFOA
 MDLNTDQE-NL
 X 10

100 110 120 130 140 150 160
 LTNNEANLIGICRICKYGDDKLIASWDGLIEVIDPRNYGDGVIAVKNLNSNNTKVKNKIPTMDTNSRLIVG
 | | :::||| ||| |: :::|: ||||
 VGTHDAPIRCVEYCP-EVNVMVTGSWDQTVKLMDPR-----TPCNAGITFSQPEKKVYTLSVSGDLIVG
 20 30 40 50 60 70

170 180 190 200 210 220 230
MNNSQVWFRLPLCEDDNGTIE---ESGLKYQIIRDVALLPKEQEGYACSSIDGRVAVEFPDDQGDDYNSSKR
 | | || | | | | | | | | |
TAARRVLVDLR---NMGYYQORRESSLKYQTRCIRAFPNKQGYVLSSIEGRVAVEYLDPSPE--VKKKK
 80 90 100 110 120 130

--More--(<space>, Q, or ? for help)

240 250 260 270 280 290 300
FAFRCHRLNLKDTNLAYPVNSIEFSPRHKFLYTAGSDGIISCNWLQTRKKIKNFAKFNEDSVVVKIACSDNLL
:||:|||| ||||:| | :|||: | :| : | : | : |
YAFKCHRLKENNLEQIYPVNAISFHNHNTATGGSDFVNTWDPFNKKRLLPVPSY-PTSIASLAFSNDGT
140 150 160 170 180 190 200

310 320 330 X
CLATSDDTPTKNAADQTIELMASSIIY--IPDYEN
|| : : || | | : | : | :
TLAIA-SSYM--YEMDDT-EHPEDGIFIRQVTDAAETKPKST
210 220 230 240

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TITLE

Lus BUB3

Project No.

Book No.

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using MMU67327 file as a probe on MASPAP, I was able to identify hu cDNAs which spanned the whole MMU sequence. Using sequencer, I took all the put ESTs and made an edited contig, removing and fixing nucleotide differences that were clear base calling errors. It was also necessary to remove one aa to fix a reading frame jump. There are 64 WD40 repeat proteins in S. cerevisiae. YET7 is closest to BUB3;

TARGET SEQUENCE FILE: huWD40 (Bub3?)

TARGET SEQUENCE TEXT:

	10	20	30	40	50	60
1	CGGACCGAGC	GATGTNNCGG	AGTAGTGGAA	ACGKIWGCTT	CTGAGGGGAG	CCCAAGATGA
61	CCGGTCTCTA	CGAGTTCAG	CTGAACGAGC	CACCCGAGGA	TGGCATCTCC	TCCGTGAAGT
121	TCAGCCOCAA	CACCTCCAG	TTCTGCTTG	TCTCTCCTG	GGACACGTCC	GTGGCTCTCT
181	ACGAITGGCC	GGCCAATCC	ATGCGGCTCA	AGTACCAGCA	CACCGCGGCC	GTCCTGGACT
241	GCGCCTTCTA	CGATCCAAGC	CATGCCTGGA	GTGGAGGACT	AGATCATCAA	TTGAAATGC
301	ATGATTGAA	CACTGATCAA	GAAATCTTG	TTGGGACCCA	TGATGCCCTT	ATCAGATGTG
361	TTGAATACTG	TCCAGAAGTG	AATGTGATGG	TCAGCTGAA	TTGGGATCAG	ACAGTTAAAC
421	TGTGGGATCC	CAGAACTCCT	TGTAATGCTG	GGACCTTCTC	TCAGCCTGAA	AAGGTATATA
481	CCCTCTCAGT	GTCTGGAGAC	CGGCTGATTG	TGGGAACAGC	AGGCCGCGAG	GTGTTGGTGT
541	GGGACTTACG	GAACATGGGT	TACGTGCAGC	AGCGCAGGGA	GTCCAGCCTG	AAATACCAGA
601	CTCGCTGCR	ACGAGCGTTT	CCAAACAAGC	AGGGTTATGT	ATTAAAGCTCT	ATTGAAGGCC
661	GAGTGGAAAT	TGAGTATTTG	GACCCAGGCC	CTGAGGTACA	GAAGAAGAAG	TATGCCCTTCA
721	AATGTCACAG	ACTAAAGAA	AATAATATTG	AGCAGATTTA	CCCAGTCAAT	GCCATTCTTT
781	TTCACAATAT	CCACAATACA	TTTGCCACAG	GTGGTTCTGA	TGGCTTTGTA	AATATTGGGG
841	ATCCATTAA	CAAAAGCGA	CTGTGCCAAT	TCCATCGGTA	CCCCACGAGC	ATCGCATCAC
901	TTGCCTTCAG	TAATGATGGG	ACTACGCTTG	CAATAGCGTC	ATCATATATG	TATGAAATGG
961	ATGACACAGA	ACATCCTGAA	GATGGTATCT	TCATTGCGCA	AGTGACAGAT	GCAGAAACAA
1021	AACCCAAGTC	ACCATGTACT	TGACAAGATT	TCATTTACTT	AAGTGOCATG	TTGATGATAA
1081	TAAACAAT	CGTACTCCCC	AATGGTGGAT	TTATTACTAT	TAAAGAAACC	AGGGAAATA
1141	TTAATTTTAA	TATTATAACA	ACCTGAAAT	AATGGAAAG	AGGTTTTTGA	ATTTTTTTTT
1201	TAAATAAAC	ACCTTCTTAA	GTGCA			

PRIMER PAIRS:

Sense

5'.GGGAGCCCAAGATGACCGGTT.3'

Anti-Sense

5'.AAATCCACCATTGGGGAGTACGAATTGT.3'

3' location: 66. Tm: 60.8°.

Size: 21 bp. GC: 62%. Base run: 3.

Mw: 6576.

Scores - Global: 64 Local: 29.

3' tetramer duplicated 8x.

3' location: 1085. Tm: 60.9°.

Size: 28 bp. GC: 43%. Base run: 4.

Mw: 8716.

Scores - Global: 80 Local: 26.

3' pentamer duplicated 3x.

Amplified Sequence Stats

Size: 1067 bp. GC: 47%.

To Page No.

Witnessed & Understood by me,

David Duhl

Date

Invented by

T. Suley

Date

Recorded by

CI

SEELEY
DECLARATION
EXHIBIT NO. 2

Project No. _____

Book No. _____

TITLE

hu BUB3 inv. disc.

From Page No. _____

Sent in interoffice mail midday [REDACTED]

Invention Disclosure

Todd Seeley

Prepared [REDACTED]

A. A novel human cDNA sequence was elucidated from existing public EST sequence data files, based on homology to Genbank acc.# U67327. U67327 is a murine cDNA sequence annotated as coding for a "WD40-repeat type I transmembrane protein A72.5". U67327 was in turn identified as a potential structural and functional murine homolog of the *S. cerevisiae* BUB3 gene.

U67327 was initially identified on the basis of a MASPARG sequence homology search using the reverse-translated *S. cerevisiae* BUB3 protein sequence as a probe. BUB3 encodes a protein involved in response to microtubule poisons in yeast. The genetic basis of sensitivity and resistance to microtubule poisons in humans is poorly characterized, and may involve a human homolog of the yeast BUB3 gene. Following identification of U67327 as a potential murine match to BUB3, the corresponding Genbank accession file was downloaded from the internet. Using either the reverse-translated *S. cerevisiae* BUB3 protein sequence or the U67327 sequence as a probe, numerous human EST sequences were identified in MASPARG homology searches which could be identified as coding for potential functional homologs of U67327 and BUB3.

These human EST sequences were compared with each other using Sequencher DNA sequence analysis software and were found to contain regions of overlap which were sufficient to define what appears to be the human homolog of the murine cDNA described in U67327. Some human EST files that were retrieved possessed annotations describing homology to yeast genes BUB3, RAE1 and YET7. The complete cDNA sequence of the human homolog of U67327 is not recorded in existing databanks, however. Human EST files were edited by aligning multiple EST sequences to determine a consensus sequence, thereby editing out what appeared to be base calling errors in individual EST files. The strategy used to compile human EST sequences into a single sequence file is attached.

The EST-derived human cDNA sequence for this gene (tentatively designated huBUB3), is as follows:

10	20	30	40	50	60	70
GCGACCGAGC	GATGTNNGCG	AGTAGTGGAA	ACGKTWCTT	CTGAGGGGAG	CCCAAGATGA	CCGGTTCTAA
80	90	100	110	120	130	140
CGAGTTCAAG	CTGAACCAGC	CACCCGAGGA	TGGCATCTCC	TCCGTGAAGT	TCAGCCCCAA	CACCTCCAG
150	160	170	180	190	200	210
TTCTCTGCTTG	TCTCTCTCTG	GGACACGTCC	GTGCGTCTCT	ACGATGTGCC	GGCCAACTCC	ATGCGGCTCA
220	230	240	250	260	270	280
AGTACCAGCA	CACCGGCGCC	GTCTGGACT	GCGCTTCTA	CGATCCAACG	CATGCCTGGA	GTGGAGGACT

2

No. _____

Witnessed & Understood by me,

[Signature]

Date

[REDACTED]

Invented by

[Signature]

Date

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Recorded by

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TITLE

LUB3

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290      300      310      320      330      340      350
AGATCATCAA TTGAAATGC ATGATTGAA CACTGATCAA GAAATCTTG TTGGGACCCA TGATGCCCCCT

360      370      380      390      400      410      420
ATCAGATGTG TTGAATACTG TCCAGAAAGT AATGTGATGG TCACTGGAAG TTGGGATCAG ACAGTTAAAC

430      440      450      460      470      480      490
TGTGGGATCC CAGAACTOCT TGTAACTCTG GGACCTTCTC TCAGCCTGAA AAGGTATATA CCCTCTCAGT

500      510      520      530      540      550      560
GTCTGCAGAC CGGCTGATTG TGGGAACAGC AGGCCGCGAGA GTGTGTTGTG GGGACTTACG GAACATGGGT

570      580      590      600      610      620      630
TACGTGCAGC AGCGCAGGGA GTCCAGCCTG AAATACCAGA CTCGCTGCAT ACGAGCGTTT CCAACACAGC

640      650      660      670      680      690      700
AGGGTTATGT ATTAAGCTCT ATTGAAGGCC GAGTGGAGT TGAGTATTG GACCCAAGCC CTGAGGTACA

710      720      730      740      750      760      770
GAAGAAGAAG TATGCCTTCA AATGTCACAG ACTAAAGAA AATAATATTG AGCAGATTGA CCCAGTCAAT

780      790      800      810      820      830      840
GCCATTTCCT TTCACAATAT CCACAATACA TTTGCCACAG GTGGTTCTGA TGGCTTTGTA AATATTGGG

850      860      870      880      890      900      910
ATCCAATTAA CAAAAGCGA CTGTGCCAAT TCCATCGGTA CCCCACGAGC ATGCATCAC TTGCCCTCAG

920      930      940      950      960      970      980
TAATGATGGG ACTACGCTTG CAATAGCGTC ATCATATATG TATGAATGG ATGACACAGA ACATCCTGAA

990      1000      1010      1020      1030      1040      1050
GATGGTATCT TCATTGCGCA AGTGACAGAT GCAGAAACAA AACCCAAGTC ACCATGTACT TGACAAGATT

1060      1070      1080      1090      1100      1110      1120
TCATTACTT AAGTGCCATG TTGATGATAA TAAAACAATT CGTACTCCCC AATGGTGGAT TTATTACTAT

1130      1140      1150      1160      1170      1180      1190
TAAAGAAACC AGGGAATAA TTAATTTTAA TATTATACCA ACCTGAAAT AATGGAAGAG ACGTTTTTGA

1200      1210      1220
ATTTTTTTTT TTAATAAAC ACCTCTTAA GTGCA

```

Because this sequence includes numerous base substitutions from U67327, this product is likely formed from the human cDNA equivalent of U67327. The predicted translation product of this sequence is almost identical to that of U67327, with few substitutions. All amino acid identities between BUB3 and the predicted human cDNA equivalent of U67327 translation product are conserved in this human sequence (designated huBUB3), as follows:

- | | |
|-----------|---------|
| 1. U67327 | (1-246) |
| 2. huBUB3 | (1-329) |
| 3. BUB3 | (1-341) |

Needleman-Wunach Alignment: (listed in Clustered order)

3

To Page No. _____

Witnessed & Understood by me,

Aurd Dahl

Date

Invented by

T. Suley

Date

Recorded by

NB89470

083

2

From f

```

U67327      1
huBUB3      1 MtgenefklnQpPaDgISvKfsPntsQfLLvsSWDtS VrlYDvPA NsmrL kyqhtg
BUB3        1 M qivqieQaPkDyISdIKilPskS ILLitSWDgSlTVyKfDiqAKNwdllQslrykh
consensus   mtge-----q-p-d-is--k-p-sq-ll--swd-sltv---d--akn---lq-----

U67327      1
huBUB3      58 avldCaFyDpT hawsG gldhqlKQHDLNTDQENLVGT HDA PIRCVEYCPENVVMVT
BUB3        58 pILcCnFiDnTdlqiyyGtvqqeILK vDLlgspsfqaITaneAnlgiCrickygdcklia
consensus   --l-c-f-d-tdl-----gt-----lkmbDLntdqnilygtahdAnpIrCveycpevnmvmt

U67327      34 GSWDQTVKIMDPR TPCNA GTFSQPEKV YTLVSVDRLIVGTaARRVLVNDL
huBUB3      114 GSWDQTVKIMDPR TPCNA GTFSQPEKV YTLVSVDRLIVGTaARRVLVNDL
BUB3        118 aSWDgIieviDPRnygdgviAvknlnSnatKVknkifTmdtnssRLIVGannsqVqwrLp
consensus   gSWDqtvklwDPRnygtpcnAvkgtfSqpEKVknkiyTlsvagdRLIVGta-rrVlVwdLp

U67327      85 RRMGYVQORRESSLKYQTRCIRAFPNKQ GYVLSSIEGRVAVE YLD PSPEVQKKKYAFK
huBUB3      165 RRMGYVQORRESSLKYQTRCIRAFPNKQ GYVLSSIEGRVAVE YLD PSPEVQKKKYAFK
BUB3        179 lceddhgtieKsgLKYQIhdvallPkeQeGYacSSIdGRVAVEffDqgddynssKrFAFr
consensus   rrmgyvqqrrESaLKYQTRCIRAFPNKQeGYvLSSIEGRVAVEfyldqpspevqkKkyAFk

U67327      143 CHRLKENNIEQIYFVMAISFHNTHNTFATGSGDGFVNIW DPFNKKRLlpvpsYPTSIALS
huBUB3      223 CHRLKENNIEQIYFVMAISFHNTHNTFATGSGDGFVNIW DPFNKKRLcqhfrYPTSIALS
BUB3        240 CHRLkldtalaYFVMAIePspRhkflyTaSGDGIscwnlqtrKkknfakfndSvvki
consensus   CHRLkennieqiYFVMAIeFhniHntfaTgSGDGFvniWndpfnKKrl-----yptSiasl

U67327      203 AFSNDGTTLAIASSYMYEMDDTEHPEDGIFIRQVTDaETKPKS T
huBUB3      283 AFSNDGTTLAIASSYMYEMDDTEHPEDGIFIRQVTDaETKPKSpotx
BUB3        301 AcS D nILclATS DPTKtnaaIdqtIelnAasiyiifdyen
consensus   AFSnDgttLaiAssymyemDDTEhpEdgIfirqvtdAetkpkS--t-n

```

B. Based on the known role of the BUB3 gene in conferring resistance and sensitivity to microtubule poisons in yeast, this human sequence has potential applications where microtubule poisons are used. An example includes chemotherapy for cancer; where microtubule poisons such as vincristine,

Witnessed & Understood by me,

David A. Hill

Date

Invented by

T. J. Kelly

Date

Recorded by

1

TITLE

hu BUB3

Project No. _____

Book No. _____

85

From P

vinblastine, taxol and taxotere are widely used. Specific cancers spontaneously acquire resistance to these anti-tumor agents, possibly through genetic or epigenetic changes which modify the activity of the human gene described here. This gene may function as a novel tumor suppressor gene or oncogene. Knowledge of this gene may therefore have important diagnostic applications in cancer treatment. Additionally, it may be possible to develop new therapeutics based on the knowledge of this gene, by identifying chemical compounds which modify the activity of this gene or the predicted gene product. Additional novel genes may also be identified on the basis of knowledge of this gene, through interactions with the predicted protein product or other strategies. These novel genes in turn could lead to new diagnostics, therapeutics and yet more novel genes.

C. This assembled human gene sequence is novel and does not match anything available in current DNA sequence data banks. The predicted translation product of this human cDNA includes an N-terminal extension which extends beyond the cDNA coding sequence reported in Genbank annotations for murine U67327. This extended region of the predicted human cDNA translation product includes additional regions of homology to the yeast BUB3 gene, and is therefore likely to be biologically relevant.

D. There has been no public disclosure of this discovery.

E. This invention was solely my own conception.

5

To Page No. _____

Witnessed & Understood by me,

David Auhl

Date

Invented by

T. Shiley

Date

Recorded by

1

NB89470

085

4

TITLE

Inv disclosure #'s-

Project No. _____

Book No. _____

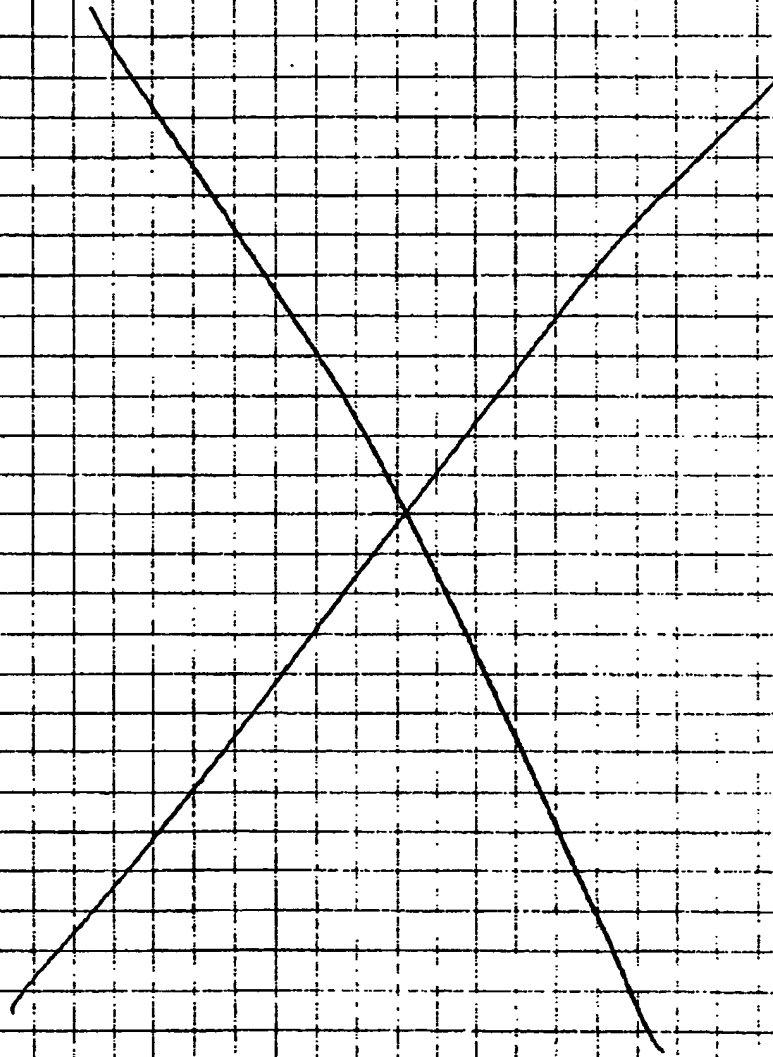
91

From Page No. _____

Ref # D90-0009

BUB3/WDA0 Ref # D-90-0008

These are ref #'s for things to add to
the files. Ling Long gave me these.



To Page No. _____

Witnessed & Understood by me,

Howard Buhl

Date

[Redacted]

Invented by

T. Luby

Recorded by

Date

[Redacted]

SEELEY
DECLARATION
EXHIBIT NO. 3

TITLE

Project No.

Book No.

87

From Base No.

TWS93

31-MER

MW AMMONIUM SALT 9719.99
MOLAR EXTINCTION AT 260nm 317100
MICROGRAMS PER OD260nm 30.6528
PICOMOLES PER OD260nm 3153.58
BASE COMPOSITION: ACGT 65811
MIXED BASES: YRNMKSWHBVDXZ 00000000000010

X=phosphate

Td (blot) .1M Na+ 66

Tm @ .1M Na+, .000001M probe 76

In TWS94

DEFINE X

Enter extinction of X, <RET> for 0: ?

TWS94

28-MER

MW AMMONIUM SALT 8640.24
MOLAR EXTINCTION AT 260nm 285600
MICROGRAMS PER OD260nm 30.2529
PICOMOLES PER OD260nm 3501.4
BASE COMPOSITION: ACGT 71064
MIXED BASES: YRNMKSWHBVDXZ 00000000000010

X=phosphate

Td (blot) .1M Na+ 70

Tm @ .1M Na+, .000001M probe 79

TWS95

22-MER

MW AMMONIUM SALT 6819.91
MOLAR EXTINCTION AT 260nm 232100
MICROGRAMS PER OD260nm 29.3835
PICOMOLES PER OD260nm 4308.49
BASE COMPOSITION: ACGT 5583
MIXED BASES: YRNMKSWHBVDXZ 00000000000010

X=phosphate

Td (blot) .1M Na+ 66

Tm @ .1M Na+, .000001M probe 73

In TWS96

DEFINE X

Enter extinction of X, <RET> for 0: ?

TWS96

29-MER

XAAATCCACCATTGGGABTACGAATTGT
MW AMMONIUM SALT 9079.55
MOLAR EXTINCTION AT 260nm 317000
MICROGRAMS PER OD260nm 28.6421
PICOMOLES PER OD260nm 3154.57
BASE COMPOSITION: ACGT 9577
MIXED BASES: YRNMKSWHBVDXZ 00000000000010

X=phosphate

Td (blot) .1M Na+ 65

Tm @ .1M Na+, .000001M probe 74

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

NB89470

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Filed 02/27/2002

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EXHIBIT NO. 3

087

From Page No. _____

PCR runs

	①	②	③	④
lan Testis meridian	2.5	2.5	2.5	2.5
AA1	1	1	1	0
DMS 93	2.5	0	0	0
94	0	2.5	0	0
95	0	0	0	0.5
96	0	0	0	2.5
Q NTP	1.75	1.75	1.75	1.75
DW	17.25	17.25	17.25	15.75
MIX 2	4.5X			
DW	19.5	22.25		
10X #2 Expand 5	22.5			
run 2	5	1.25		
			94° 21'	
			94° 15' 40.3" 68° 4' X30	
			10° 68° C	
			4°C	

Run on -

Rapid Lig

1

2

3

4

BUB3 run

Lig

289

290

291

PCR 31 17

Mix 32

QNA dit 12

2x lig 52

lig 17

The RT;

TF to XL (Blue plate to Kan / kg)

1 2 3 4

Gel Results - Kinda crappy;
 tho BUB3 looks great
 Try with double oligos
 mu/dur; sort
 nested PCR?!

To Page No. _____

Witnessed & Understood by me,

Ward

Date

Invented by

T. Guly

Date

Recorded by

4

From Page No. _____

PER runs

	①	②	③	④
Lu Testis monochor	2.5	2.5	2.5	2.5
AA1	1	1	1	0
rw593	2.5	0	0	0
94	0	2.5	0	0
95	0	0	0	0.5
96	0	0	0	0.5
Q NTP	1.75	1.75	1.75	1.75
DW	19.25	19.25	19.25	15.75

Mix 2 4.5x
 DW 19.5 87.75
 10x#2 Expand 5 22.5
 2x12 .5 1.25

Proj 30 94' 2'
 26 94' 15" 60' 3" 68' 4" x 30
 31 10' 68"
 12 40'

8am on -

2/25/97 rapid log

1

2

3

4 BUB3 line

Lig

289

290

291

PER 12

Mix 12

ONADIA 12

2x Lig 53

Lig 17

1hr RT;

T.F. to XL-1 Blue plate to team / top

1/23/97

Gel Results - Kinda Crappy;
 Also BUB3 looks great!
 Try with double plates
 mul/blue; Sorta
 nested PCR?!

To Page No. _____

Witnessed & Understood by me,

Date

Invented by

T. Suley

Recorded by

4

Date

SEELEY
DECLARATION
EXHIBIT NO. 4

TITLE

BUB3

Project No.

Book No.

93

From Page No. 88

Reg 289-291 : 1000's colonies on cell, lawn.

1 inoc 8 ea 289, 291 LB/flu Kan 3 ml ea

1 inoc 245 ml for lg scale prep.

pm. used turbid cultures to

inoc 500 ml LB with

also from EGFP - BAC stock.

on 5 289-291 very slow growth -

Remoc fresh LB. maybe a problem with media or temp control on shaker.

Got a "mega" QiaGen vol from Duhr lab. and resp in 1 ml DW and passed to Ling for transfection studies.

Note also Ling has successfully generated

Sense
Anti sense
Sense
Anti sense

And grew up lg scale preps for TF. We are ready to start, except that the cells we requested from Eve Mendoga are either not here yet or growing exceptionally poorly (MCF.7)

2/28 Ling will do screens on 289-291

289

290

291 in BUB3

To Page No.

Witnessed & Understood by me

David Dull

Date

Invented by

Thulay

Recorded by

C

Date

NB89470

Serial No. 10/084,700

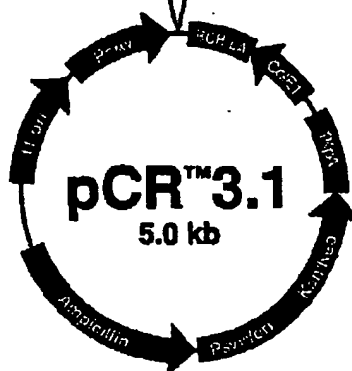
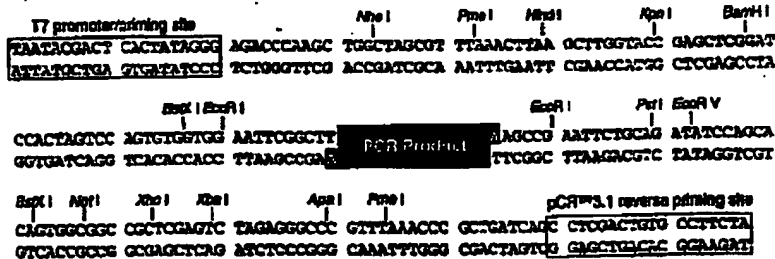
Filed 02/27/2002

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EXHIBIT NO. 4

093

From Page No. _____



Appendix B: Additional figures ... continued

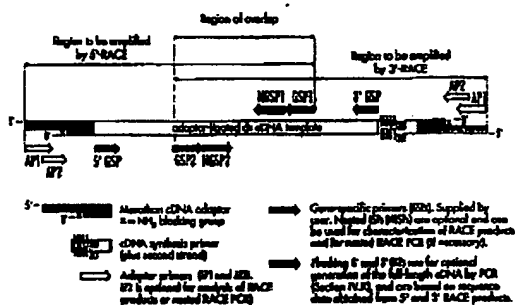


Figure 6: The template and primers used in Marathon RACE reactions and amplification of the full-length cDNA. Marathon-ready cDNAs have the general structure depicted above. Each side of Marathon-ready cDNA is essentially a library of ordered cDNA fragments which can amplify many different cDNAs using different sets of gene-specific primers (GSPs). Although the positions of nested genes and adapter-specific primers are shown, nested PCR is not necessary for successful Marathon RACE reactions in most cases. For more information on primer design, see Section IV.B.

The primers shown above will create overlapping 5' and 3' RACE products, which can subsequently be joined by restriction digestion and ligation to create the full-length cDNA (provided they are suitable restriction sites in the region of overlap). Using primers designed to give overlapping RACE products also means that these primers can be used together to generate the overlapping fragments if the overlap is at least 100-200 bp. The primers provided control for PCR. However, it is not strictly necessary to use primers having overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. The primers themselves can overlap (i.e., be complementary).

Note of the Marathon cDNA Adapter in RACE reactions: The specificity of Marathon RACE reactions is greatly enhanced by the presence of an AP1 binding site on the adapter-linked cDNAs. This site is created on the cDNA of interest by extension from the inner, gene-specific primer during the first RACE cycle, as shown in Figure 7 on the previous page. The AP1 primer on the Marathon cDNA adapter binds to the 3' end of the adapter-linked cDNA, and the gene-specific primer binds to the AP1 binding site on the cDNA of interest. The Marathon Adapter is also designed for expression PCR (Figure 8). Together, these features result in more efficient RACE amplification of the desired product, with a lower level of background.

Appendix B: Additional figures ... continued

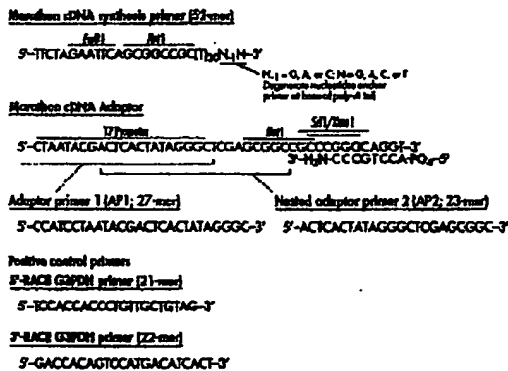


Figure 6: Sequences of the Marathon cDNA Synthesis Primer, the Marathon cDNA Adapter, the AP1 and AP2 primers, and the positive control GSPDH primers. The GSPDH primers bind to a region of the gene that is identical in human, mouse, and rat.

To Page No. _____

Witnessed & Understood by me

David Duhal

Date

Invented by

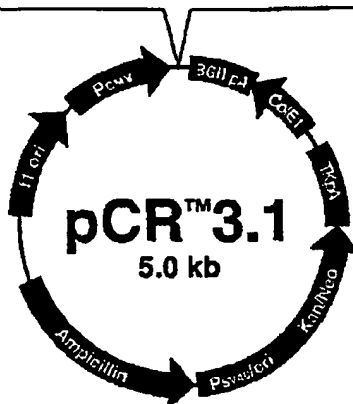
T. Riley

Date

Recorded by

"

T7 promoter:anning site
 TAATACGACT CACTATAGGG AGACCCAGC TGCTAGCGT TTAAGTAA GCTTGGTACC GAGCTCGGAT
 ATTATGCTGA TGTATATCCC TCTGGGTTCC ACCGATCCGA AATTGGAATT CGACCCATCC CTCGAGGCTA
 BstX I EcoR I
 CCACATGTC CAGTGGGTGG AATTCCGGCTT PCR Product AAGCCG AATTCTGCAG ATATCCAGCA
 GGTGATCAG TCAACACCAC TTAAGCCGA TCTGGC TTAGACGTC TATAGCTCT
 EcoR I Pst I EcoR V
 BstX I NotI XhoI XbaI AgeI PstI pCR™3.1 reverse priming site
 CAGTGGGGCG CGCTCGATG TAGAGGGGCC GTTTAAACCC GCTGATACG CTCGACTGG CCGTCTTA
 GTCAACGCC CGAGAGTCG ATCTCCGGCC CAAATTGGGG CGACTAATCG GAGCTCGAC CGAGATG



Appendix B: Additional figures ... continued

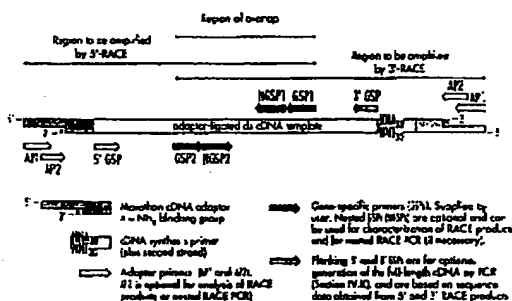


Figure 8: The templates and primers used in Marathon RACE reactions and amplification of the full-length cDNA. Marathon-Ready cDNAs have the generic structure depicted above. Each tube of Marathon-Ready cDNA is essentially a library of uncloned cDNA from which you can amplify many different cDNAs using different sets of gene-specific primers (GSPs). Although the positions of nested gene- and adapter-specific primers are shown, nested PCR is not necessary for successful Marathon RACE reactions in most cases. For more information on primer design, see Section IV B.

The primer shown above will create overlapping 8' and 3' RACE products, which can subsequently be joined by restriction digestion and ligation to create the full-length cDNA (provided there are no alternative splicing sites in the region of overlap). Using primer design to create overlapping RACE products is a useful technique to generate overlapping fragments for the purpose of cloning a cDNA if the overlap is at least 100–200 bp. This provides a useful control for PCR. However, it is not absolutely necessary to use primers that give overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. The adjacent sequences can overlap (i.e., be complementary).

Role of the Marathon cDNA Adaptor in RACE reactions: The specificity of Marathon RACE reactions is greatly enhanced by the absence of an AP binding site on the adaptor-spliced cDNA template. This is achieved by exclusion of adapter sequences from the first round PCR cycle, shown in Figure 6. The resulting cDNA template contains the Marathon cDNA Adaptor flanking extension of the 5' end of the adapter-bound cDNA, and thus prevents formation of an AP binding site on the general population of cDNAs. The Marathon Adaptor is also designed for suppression (PCR S-B). Together, these features result in more efficient RACE amplification of the desired product, with a higher level of background.

Marathon cDNA synthesis primer (52-mer)

$$5'-\text{TTCTAGAAATTCAGCGGCCGCT}_{100}\text{N}_1\text{N}-3'$$

$\text{N}_1 = \text{G, A, or C} = \text{N-G, A, C, or}$
 Degrades nucleotides and/or primer at base of poly-A tail

Marathon cDNA Adaptor

¹⁷ French ^{54/85.} Borl
 5'-CTAATACGACTCACTAAGGGCTGAGCGCGCGCCGGCAGGT-3'
 3'-B₂NCCCGTCCAP₀5

Adaptor primer 1 (AP1; 27-mer)

5'-CCATCTAATACGACTCACTATAGGGC-3' 5'-ACTCACTATAGGGCTCGAGCGGC-3'

Positive control primer

5'-RACE GAPDH primer (21-mer)

5-3CCACCACCCCTGTTGCTGTAG-3'

3-10-2008 10:01:01

3'-RACE GSPDH primer (22-mer)

5'-GACCACAGTCCATGACATCACT-3'

Figure 2: Sequences of the Marathon cDNA Synthesis Primer, the Marathon cDNA Adaptor, the AP1 and AP2 primers, and the positive control G3PDH primers. The G3PDH primers anneal to a region of the gene that is identical in human, mouse, and rat.

Write in 10 days letter
have next 2 weeks, and accept 7 for a 10 mark from

[illegible]

David Dink

NB89470

094a

SEELEY
DECLARATION
EXHIBIT NO. 5

TITLE

lm BUD3 analysis

Project No. _____

Book No. _____

17

From Page No. _____

289 5' RACE

290 5' RACE

291 lm BUD3

Todd did PCR and ligation

289 8.200 1 vector control

Digest with EcoRI

289 1-8

290 1-8 digest with NotI

Result: all are vectors

291 1-8
291 1-8
291 1-8
291 1-8
291 1-8

Digest with Acp718, all are sense orientation

Pick up more 291 clones

To Page No. _____

Witnessed & Understood by me,

[Signature]

Date

Invented by

Date

Recorded by

[Signature]

NB92100

Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 5

017

TITLE

[redacted] Lin BIRD3 analysis

Project No. _____

Book No. _____

17

From Page No. _____

[redacted]

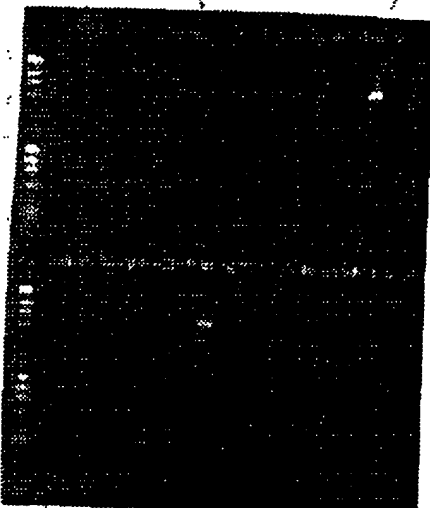
289 3' RACE

290 5' RACE

291 Lin BIRD3

Totaled did PCR and ligation

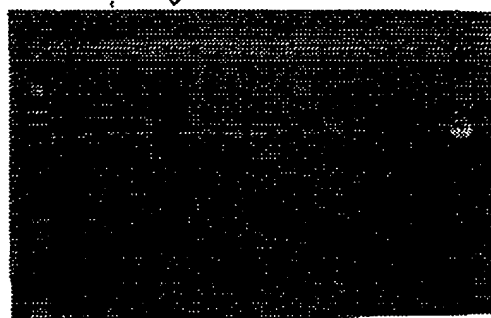
289 — 8.100 — 8 — vector control



Digest with EcoRI

289 - 8

290 - 8 Digest with NotI



Result: all are wetted

291

1 — 8

291.2
291.3
291.6
291.7



Digest with Acp215, all are some initiation

Pick up more 291 clones

To Page No. _____

Witnessed & Understood by me.

X [signature]

Date

[redacted]

Reviewed by

Recorded by

[signature]

Date

[redacted]

NB92100

017a

Project No. _____

Book No. _____

TITLE BUB-3 analysis Remit.

From Page No. _____

291-9

291-13

291-16

291-18

291-21

291-28
vector

291-9 → 291-30 directly

Asp718

~~291-13~~
polyet 291-13, 291-16, 291-18

291-21, 291-26, 291-27

291-28, 291-30 with Not I

to conform the orientation
of the insert.

No.	A1	A2	A1-A2	A1/A2
1	0.000	-0.004	0.004	0.0000
2	0.211	0.110	0.101	1.9222

2.11-8/λ

— large scale prep 291-2 and send to FASTrack sequence.

1 -0.000 0.005 -0.005 -0.0230

2 0.100 0.105 0.003 1.7854 291-2

1.58-8/λ

To Page No. _____

Witnessed & Understood by me,

Date

Invented by

L. Wang

Recorded by

Date

Project No. _____

Book No. _____

TITLE

BUB-3 sample's Remit.

From Page No. _____

291-9

291-13

291-16

291-26

291-27

291-30

291-30

291-9 → 291-30 digest by

Asp 718

~~291-13~~

digest 291-13, 291-16, 291-18

291-21, 291-26, 291-27

291-28, 291-30 with Not 1

To conform the volatility
of the insert.

No. A1 A2 A1-A2 A1/A2

1 0.000 0.004 0.004 0.0000

2 0.211 0.110 0.101 1.9222 EGPA

2.11-8/2

Large scale prep 291-2 and send to FASTrack sequencer

1 -0.000 0.005 -0.005 -0.0238

2 0.100 0.105 0.003 1.7054 291-2

1.58-8/2

To Page No. _____

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

SEELEY
DECLARATION
EXHIBIT NO. 6

Project No. _____

Book No. _____

TITLE

hUB3.

From Page No. _____

Footnote data

291-2 sequence: PCR 31 vector

T7 = TWS 95 end (start codon overlaps TWS 95)

JET 1521 = TWS 96 end.

T7 - found all nt matches to beyond nt 333 of BUB sequence.

1521. " all nt " " " nt 330.

→ Great sequence;

294 sample failed?!

To Page No. _____

Witnessed & Understood by me,

David Dahl

Date

Invented by

Suley

Date

Recorded by

n

SEELEY
DECLARATION
EXHIBIT NO. 7

From Page No. _____

last week, ^{used} inoc 16 new BUB3 pos clones (291-ly)
 Since we still need an antisense clone.

These were to be harvested last Thurs, but not
 growing well at end of day. Trans. to new
 flasks. For growth, harvested pellets
 and froze pellets for mRNA prep; Cultures to VC

uninoculated 291 31-46. Plotted 100% DW.

dis ea EcoRI
 Asp718 (separately)

DNA 5
 DW 12
 10x H₂O 2
 EcoI (or Asp) 1

X18
 1216
 36
 18
 15 dig → 2 mixes up
 for ea one.

dig ~ 2h; run 1% gel.

Results - Great. 9/16 have
 BUB3 insert by EcoRI.
 One has slightly smaller
 insert - poss false variant?

2 of each BUB3 each
 Orient. picked. Asp718
 ~ 200 nt for lg scale prep
 = 291-45 max. 150 ml
 Can - LB overnight.

Qiagen midiprep.
 resp pellet 2502 DW.

To Page No. _____

Witnessed & Understood by me,

Barred Dahl

Date

Invented by

Whaley

Recorded by

Date

Page No.

last week, ^{wed} inc 16 new BUB3 perclones (291-ly)
 Since we still need an amplicon clone.

These were to be harvested last Thurs, but not
 growing well at end of day, Thurs. At new
 shaker. For growth, harvested pellets
 and froze pellets for min prep; cultures to VC

miniprep 291 31-46. Skipped 100% DW.

dig ea Euc

Asp 718 (separately)

DATA 5
 3W 12.5
 10x4 (or 8) 2
 EcoRI (or Asp) 1

K18
 216
 3/8
 18

2 mixes one
 for ea enz.

15/dig

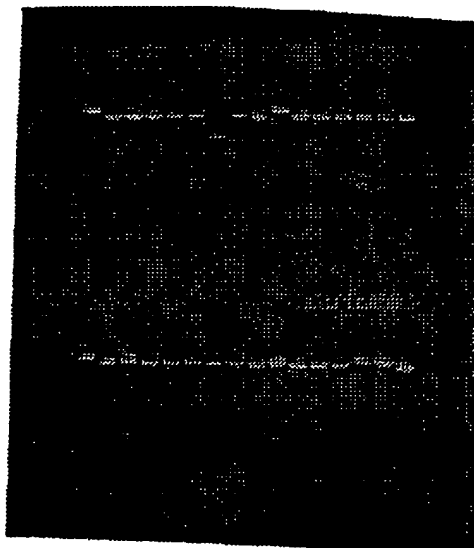
dig - 2h; run in gel.

Results - great. 9/16 have
 BUB3 insert by EcoRI.
 One has slightly smaller
 insert - possible variant?

2 of each BUB3 each
 Orient. picked Asp 718
 - 200 nt for lig scale prep

= 291-45 inc. 160 ml
 Kan-LB overnight.

Qiagen midiprep
 Asp pellet 250% DW.



To Page No.

Witnessed & Understood by me

David Aubl

Date

[Redacted]

Invented by

Disley

Recorded by

11

Date

[Redacted]

Project No. _____

Book No. _____

TITLE BUB 3 & Lense

From Page No. _____

Also want BUB3 probe for Northern -
 So dig EcoRI New 291-45 prep

EcoRI DNA 10
 MW 75
 10x HA 10
 EcoRI 5
 100

Start 3:40
 Stop 4:45 p
 Run on 20V
 12 format

Stained gel, Cut out
 band - looks great!
 put to -20°C prep later

To Page No. _____

Witnessed & Understood by me.

David Duhl

Date

Invented by

i. Luley

Date

Recorded by

Project No.

Book No.

TITLE

BUB 3 & Sense

From Page No.

Also want BUB3 probe for Northern -
 so dig EcorI New 291-45 prep

EcorI DNA 10

BW 75

10x H 10

Eckbi 5

100

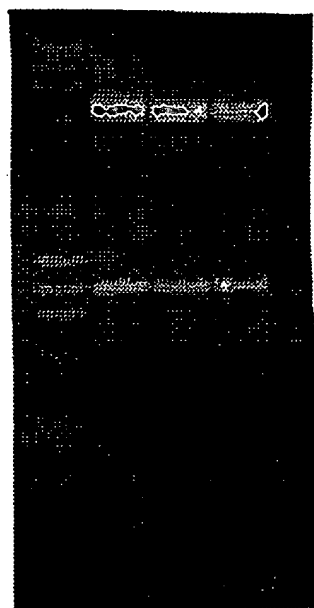
Start 3:00

Stop 4:45p

run on 20V

Lg ferment.

Stained gel, cut out
 band - 100bp frag!
 put to -20°C prep lab



Page No.

Witnessed & Understood by

David Buhl

Date

Initialed by

Recorded by

Lulley

SEELEY
DECLARATION
EXHIBIT NO. 8

TITLE

Probe northern BUB3

Project No. _____

Book No. _____

157

From Page No. 139, 134.

Stripped blot of BUB1 probe (low signal anyway)

Quinquick gel prep'd frag 291-45 pg 136;
eluted w/ total (2 cols, 500 µl ea)

prehyb blot 12:20p.

Probe: ~1 kb - BUB3 2st 150ng/2
3 2 to gel

for 5 ng/2 did 1:30 dil;

2 2 = 10ng put to 45 2 2
Boil heat 100°C 5 min to denat
used to keep "readyprime"

+ 5 2 3rd dCTP
12:45 start labelling; 1 hr ~ 25 min
Chromaspin column to remove XS label

probe heat 100°C 5 min to denat
add to fresh hyb start blot hyb 1:30

2:30 start washes as per pg 139

Results: bands! 3 species by size
mobility 4.0 kb, 2.7, 1.4.

1 4 was expected. All bands in all tissues;
But TESTIS greatly enriched for
1.4 kb message. I am interested
in these other messages.

To Page No. _____

Witnessed & Understood by me,

David Dahl

Date

Invented by

T. D. Wiley

Date

Recorded by

Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 8



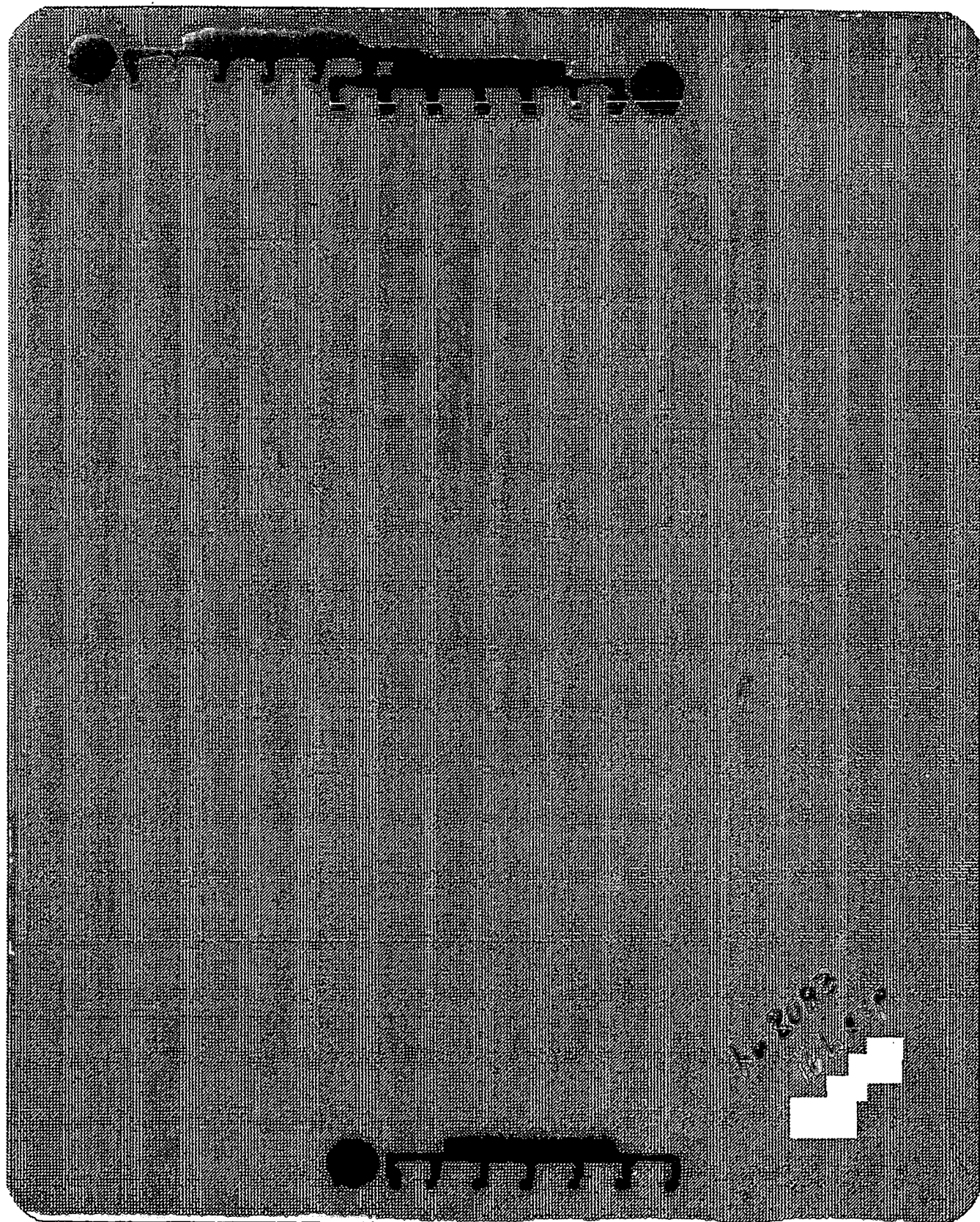
NOTEBOOK # 8947

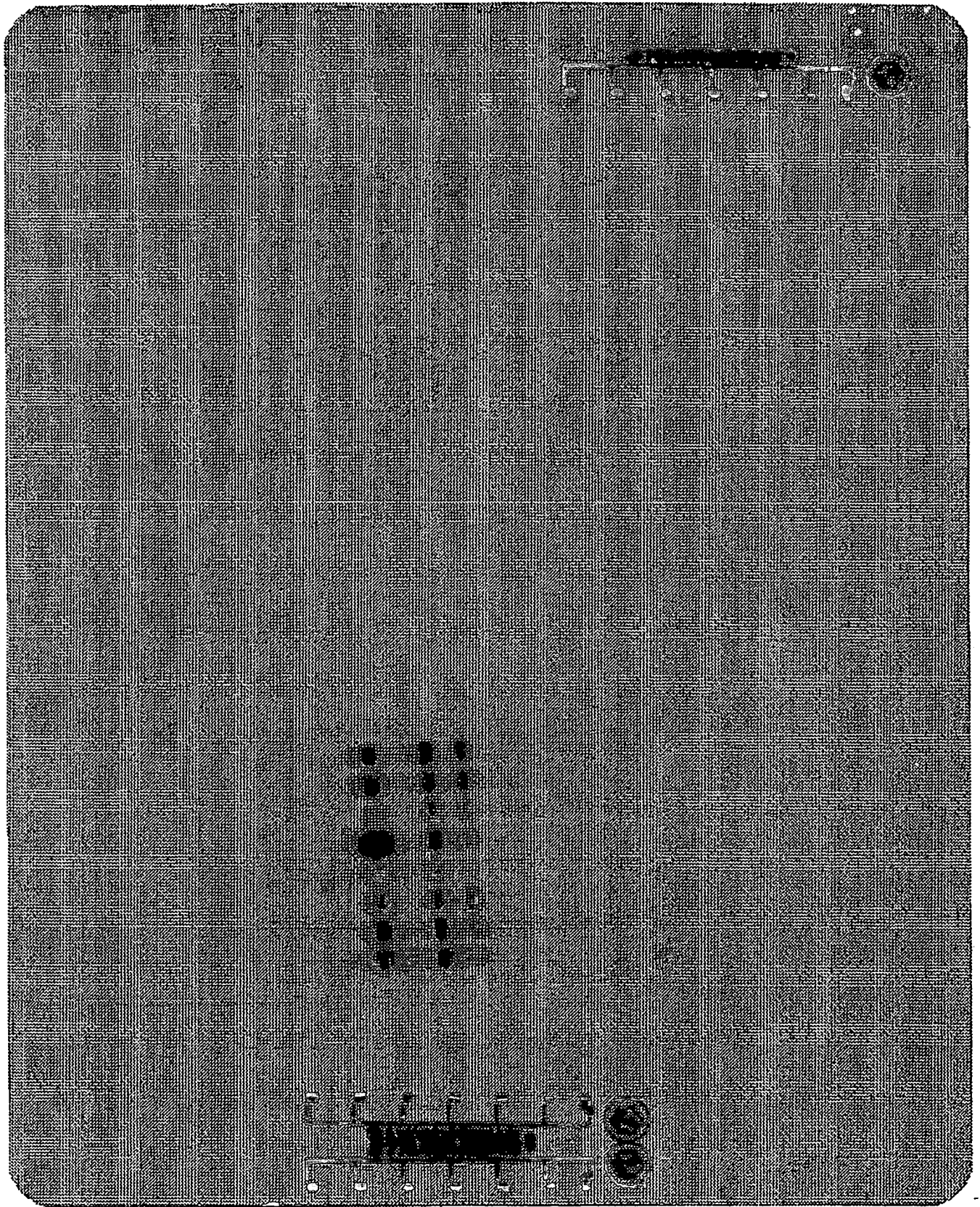
PAGE # _____

DATE: _____

NAME: Todd Seeley

NOTES: 2 x ray films; Clontech
MTN II blot huBUB3 probe





Project No. _____

Book No. _____

TITLE

Northern

From Page N

PRODUCT: Human Multiple Tissue Northern (MTN™) Blot II**CATALOG #:** 7759-1**LOT #:** 7010569**STORAGE CONDITIONS:**

- Store unused blot at room temperature (22°C) sealed in a plastic bag away from light.
- Store control probe at -20°C.
- Store ExpressHyb™ at room temperature (22°C). If a precipitate forms, warm to 68°C and mix gently before use.

SHELF LIFE:

1 year from date of receipt under proper storage conditions.

SHIPPING CONDITIONS:

Room Temperature (22°C)

FOR RESEARCH USE ONLY

DESCRIPTION: Northern blot containing approximately 2 µg of poly A⁺ RNA per lane from eight different human tissues. RNA is run on a denaturing formaldehyde 1.2% agarose gel, transferred to a charge-modified nylon membrane by Northern blotting, and fixed by UV irradiation. Lanes 1-8 contain, in order, RNA from human spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte. RNA size marker bands are indicated in ink in the left margin of the blot. A notch has been cut from the lower left-hand corner of the membrane to provide orientation (see diagram below). Note: RNA is immobilized on the opposite face of the membrane.

PACKAGE CONTENTS:

- Human MTN Blot II
- 100 ng Human β-actin cDNA control probe
- 25 ml ExpressHyb™ Hybridization Solution
- Complete User Manual (PT1200-1)

POLY A⁺ RNA SOURCE: The age and sex of tissue donors may vary, but all tissue is, as far as can be determined, free of disease.

QUALITY CONTROL DATA

1. All poly A⁺ RNA is examined by denaturing gel electrophoresis to ensure intactness of RNA.
2. When the production lot of any of the poly A⁺ RNAs used to prepare the blot changes, a representative blot is probed with a radioactively labeled human β-actin cDNA control probe. The presence of a single 2.0-kb band in all lanes is confirmed.

kb
9.5
7.5
4.4
2.4
1.35

1 2 3 4 5 6 7 8

• • • • • • • •

spleen
thymus
prostate
testis
ovary
small intestine
colon (mucosal lining)
peripheral blood leukocyte

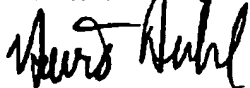
REFERENCE

1. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Diagram of MTN Blot

to Page No. _____

Witnessed & Understood by me.



Date



Invented by



Recorded by



Date



SEELEY
DECLARATION
EXHIBIT NO. 9

TITLE

Patent only

Project No. _____

Book No. _____

159

From

Inv. Discl. D-90-0008, D-90-0009

T. Seeley

2. huBUB3 (D-90-008).

- Reverse translated yeast BUB3 resulted in multiple MASPAP homology hits with ESTs of murine and human origin.
- Murine EST hits were all related to a single murine cDNA entry (Genbank#U67327).
- This entry predicts a protein with a G-beta repeat (WD40) structure, found in known signaling proteins.
- Human EST homologs to this murine sequence were collected and combined with the aid of Sequencher software to create a contiguous human cDNA sequence.
- The predicted human and murine proteins are closely related.
- The contiguous human cDNA sequence is novel, and predicts a protein with structural homology to BUB3.
- PCR primers designed from this sequence produced a product from a human testis cDNA pool of the expected size.
- The DNA sequence of the ends of this PCR fragment matched the predicted human cDNA sequence.
- A human mRNA of the expected size (~1.4 Kb) is expressed at low levels in tissues from spleen, thymus prostate, ovary, small intestine, colon mucosa and peripheral blood leukocytes.
- This human mRNA is significantly over-expressed in adult testis, a tissue with high levels of cell proliferation.
- Two other mRNAs (~2.7 and ~4.0 Kb) were expressed at low levels in all tissues examined.
- Knowledge of this sequence may have applications in diagnostic and therapeutic applications.

To Page No. _____

Witnessed & Understood by me,

David Duh

Date

Invented by

T. Seeley

Date

Recorded by

NB89470

Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 9

159

SEELEY
DECLARATION
EXHIBIT NO. 10

TITLE

hub3

Project No.

Book No.

175

From Page No.

While I was away, had Clum find
sequence 291-2 hub3 sense clone giving
her the predicted sequence as a reference.
291-2 was a perfect match to the reference
except for a "C". At this position, the
reference sequence was divergent with
some ESTs specifying "A", some "N" and
one "C". I decided to make it an A in
the reference since more ESTs had A than
others.

Alignment received [redacted], printed [redacted].

SEQUENCE NAMES, THEIR LENGTHS and % ALIGNMENTS

# 1:	hub3.ref	1068	99.81%
# 2:	291-2	1089	97.89%

In alphabet in which alignment was found:

0	{GGGAGCCCAAGATGACCGGTTCTAACGAGTCAAGCTGAACCAGCCA
0	gaattoggett	{GGGAGCCCAAGATGACCGGTTCTAACGAGTCAAGCTGAACCAGCCA
47		CCCGAGGATGGCATCTCCTCCGTGAAGTTCAGCCCCAACACCTCCAGTTCCTGCTGTGTC
58		CCCGAGGATGGCATCTCCTCCGTGAAGTTCAGCCCCAACACCTCCAGTTCCTGCTGTGTC
107		TCCTCCTGGGACACGTCGGTGCCTCTACGATGTGCGGCCAACTCCATGCGGCTCAAG
118		TCCTCCTGGGACACGTCGGTGCCTCTACGATGTGCGGCCAACTCCATGCGGCTCAAG
167		TACCAGCACACCGGCGCGTCTGGACTGCGCCTTCTACGATCCAACGCATGCGCTGGAGT
178		TACCAGCACACCGGCGCGTCTGGACTGCGCCTTCTACGATCCAACGCATGCGCTGGAGT
227		GGAGGACTAGATCATCAATTGAAAATGCATGATTTGAACACTGATCAAGAAAATCTTGT
238		GGAGGACTAGATCATCAATTGAAAATGCATGATTTGAACACTGATCAAGAAAATCTTGT
287		GGGACCCATGATGCCCCATATCAGATGTGTTGAATACTGTCCAGAGTGAATGTGATGGTC
298		GGGACCCATGATGCCCCATATCAGATGTGTTGAATACTGTCCAGAGTGAATGTGATGGTC
347		ACTGGAAGTTGGGATCAGACAGTTAACTGTGGGATCCAGAACTCCTTGTATGCTGGG
358		ACTGGAAGTTGGGATCAGACAGTTAACTGTGGGATCCAGAACTCCTTGTATGCTGGG
407		ACCTTCTCTCAGCCTGAAAAGGTATATACCTCTCAGTGTCTGGAGACCGGCTGATTGTG
418		ACCTTCTCTCAGCCTGAAAAGGTATATACCTCTCAGTGTCTGGAGACCGGCTGATTGTG
467		GGACAGCAGGCCCGCAGAGTGTGGTGTGGGACTTACGGAACATGGGTTACGTGCAGCAG
478		GGACAGCAGGCCCGCAGAGTGTGGTGTGGGACTTACGGAACATGGGTTACGTGCAGCAG

To Page No.

Witnessed & Understood by me:

David Dahl

Date

Invented by

T. Hulley

Date

Recorded by

"

NB89470

Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 10

175

Project No. _____

Book No. _____

TITLE

mu BUB3

From Page No. _____

527 CGCAGGGAGTCCAGCCTGAAATACCAGACTCGCTGCATACGAGCGTTTCCAAACAAGCAG

538 CGCAGGGAGTCCAGCCTGAAATACCAGACTCGCTGCATACGAGCGTTTCCAAACAAGCAG

587 GGTATGTATTAAGCTCTATTGAAGGCCGAGTGGG a. [AGTTGAGTATTGGACCCAAG

598 GGTATGTATTAAGCTCTATTGAAGGCCGAGTGGG c. [AGTTGAGTATTGGACCCAAG

643 CCTGAGGTACAGAAGAAGAAGTATGCCTTCAAATGTCACAGACTAAAAGAAAATATAT

654 CCTGAGGTACAGAAGAAGAAGTATGCCTTCAAATGTCACAGACTAAAAGAAAATATAT

703 TGAGCAGATTACCCAGTCAATGCCATTCTTTTACAAATATCCACAATACATTGGCCAC

714 TGAGCAGATTACCCAGTCAATGCCATTCTTTTACAAATATCCACAATACATTGGCCAC

763 AGGTGGTTCTGATGGCTTTGTAAATATTTGGGATCCATTAAACAAAAGCGACTGTGCCA

774 AGGTGGTTCTGATGGCTTTGTAAATATTTGGGATCCATTAAACAAAAGCGACTGTGCCA

823 ATTCCATCGGTACCCACGAGCATCGCATCACTTGCCTTCAGTAATGATGGGACTACGCT

834 ATTCCATCGGTACCCACGAGCATCGCATCACTTGCCTTCAGTAATGATGGGACTACGCT

883 TGCAATAGCGTCATCATATATGTATGAAATGGATGACACAGAACATCCTGAAGATGGTAT

894 TGCAATAGCGTCATCATATATGTATGAAATGGATGACACAGAACATCCTGAAGATGGTAT

943 CTTCAATCGCCAAGTGACAGATGCAGAAACAAAACCAAGTCACCATGTACTTGACAAGA

954 CTTCAATCGCCAAGTGACAGATGCAGAAACAAAACCAAGTCACCATGTACTTGACAAGA

1003 TTTCATTTACTTAAGTGCCATGTTGATGATAATAAAACAATTCGTACTCCCAATGGTGG

1014 TTTCATTTACTTAAGTGCCATGTTGATGATAATAAAACAATTCGTACTCCCAATGGTGG

1063 ATTT] 1.....

1074 ATTT] aagcogaattc

291 log EVAVE → matches SC BUB3; mu BUB3

To Page No. _____

Witnessed & Understood by me,

David Dahl

Date

Invented by

T. Hulley

Recorded by

Date

SEELEY
DECLARATION
EXHIBIT NO. 11

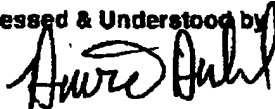
From Page No. _____

prepared stuff for patent. Noted that an important part of my argument that these should be important genes should include fact that BUB2 is related to the oncogene. I remember reading the proto-onc gene (c-fos) has not been described. I thought the human counterpart of mouse tbc-1 should be an excellent candidate. Many human ESTs give hits on tbc1. I have assembled these into a contig and ordered primers to get RACE and internal products.

OK, following is ^{1st} the legal docs I presented and addenda to prev. Inv disclosures which have raw sequence data, and then the oligos. I think a tbc homolog sounds fun to pull out esp since not completely represented in EST bank - gaps in coverage.

To Page No. _____

Witnessed & Understood by me,



Date



Invented by

T. Bailey

Date



Recorded by

u

TITLE

Patent Mktg

Project No. _____

Book No. _____

31

From Page No. _____

Yeast spindle defect signaling:Spindle
Defects,
PoisonsG2 Cell
Cycle
Control

Gene	Function	Hu/Mu Homolog	Expression
<i>MPS1</i>	essential kinase, "MonoPolar Spindle", spindle protein	ttk(hu), esk(mu)	testis, thymus, BM, embryo
<i>BUB1</i>	kinase, binds BUB3		
<i>BUB3</i>	binds BUB1, MAD3, C-beta (WD40) repeats		
<i>MAD2</i>	kinetochore protein	hMAD2	HeLa
<i>MAD1</i>	coiled-coil motif, kinased in G2 in response to stim.		
<i>MAD3</i>	binds BUB3		
<i>BUB2</i>	dispensable for ctf13 delay	tre-2, bcl1	testis (kidney)

To Page No. _____

Witnessed & Understood by me,

David Dahl

Date

Invented by

Saley

Recorded by

Date

Human and murine homologs of *scBUB3*

340 350

scUB3 T I L N A S S Y I F Y P C T
huBUB3
muBUB3 T

To Page No.

Patent way

Page No.

Human and murine homologs of *scBUB3*

<i>scBUB3</i>	M Q I V O M E A K Y N D I I I - S K S L R	10	20	30
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		40	50	60
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		70	80	90
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		100	110	120
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		130	140	150
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		160	170	180
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		190	200	210
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		220	230	240
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		250	260	270
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		280	290	300
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		310	320	330
<i>huBUB3</i>				
<i>muBUB3</i>				
<i>scBUB3</i>		340	350	
<i>huBUB3</i>				
<i>muBUB3</i>				

Amir Dadi



Tamara

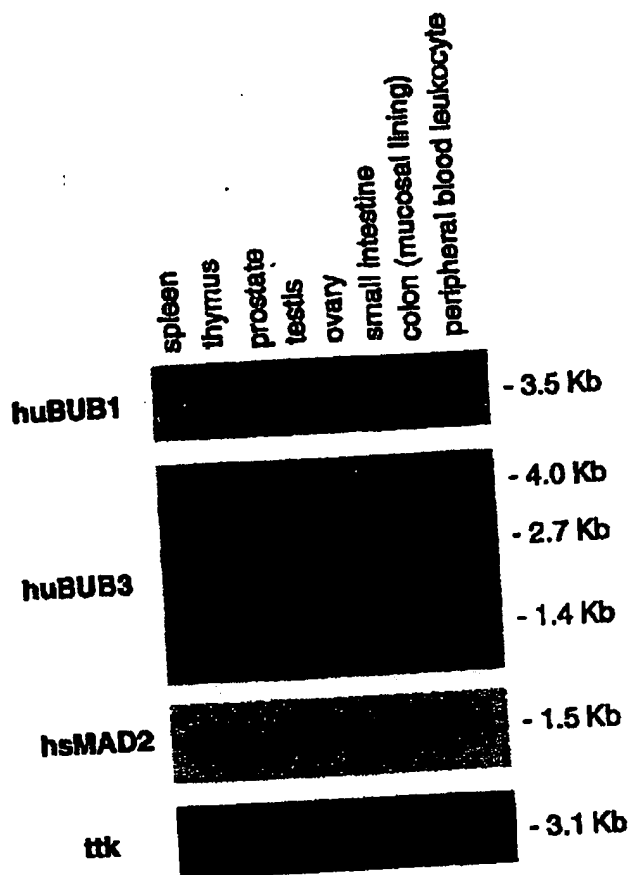


Human and murine homologs of *scBUB3*

<i>scBUB3</i>	M 0 1 V 0 T E 0 A 2 K 0 Y 1 5 D 1 K 1 1 F 1 S K N L 3	10	20	30
<i>huBUB3</i>	M T C G S N P F K I N O F E R D G I S S V K P S P N T S T O F L			
<i>muBUB3</i>	M T C S N E F K I L N O F P E D G I S K A K Y S P N T S Q F L			
<i>scBUB3</i>	E I T E W D G S L I V K E D I O A K N V D I I U S L R V K	40	50	60
<i>huBUB3</i>	E V S S W D P E S W R L D V A L A N D			
<i>muBUB3</i>	L V S S W D T S V R L V R E P A N S			
<i>scBUB3</i>	E P L E C C N F I N F I O I Y V G T Y G E T L K V	70	80	90
<i>huBUB3</i>	E T G A V L D C A Y A D R L			
<i>muBUB3</i>	E T G A V L D W A F F E D P L			
<i>scBUB3</i>	D S I G S P S F Q A L I N N E N G I C R I K Y G D D K	100	110	120
<i>huBUB3</i>	D E N T D Q E N L G G I D A P L C A V E Y S P A N V			
<i>muBUB3</i>	D E N T D Q E N L G G I D A V L T O W E A C P E A N V			
<i>scBUB3</i>	L I A A S W D G L I E V I D E N Y G H G V I A V K N L N S	130	140	150
<i>huBUB3</i>	L T P C G Y D I O T E S V D E R			
<i>muBUB3</i>	M A T E S W D Q L A V E S V D E R			
<i>scBUB3</i>	N N T K V K N K I F E N D T N S S P A I V A M N S S O R W	160	170	180
<i>huBUB3</i>	E T P S O P E S K A S G D R L E Y E A G R E S A Y			
<i>muBUB3</i>	E N T S O P E S K A S G D R L E Y E A G R E S A Y			
<i>scBUB3</i>	F R L P L C E D D N K T I E S G E K Y Q I D V A L L E K	190	200	210
<i>huBUB3</i>	E N L A V A C G W D D Q R R A N S E K K D R C R A L F P N			
<i>muBUB3</i>	E D L R N M I C A Y E Q O R R E S S K C Q R R C R A L F P N			
<i>scBUB3</i>	E Q E G G A C S P D I R V A C K F F P D Q G D Y N S S K	220	230	240
<i>huBUB3</i>	E Q - C A V A C L E D E S S P - - V A Q P K			
<i>muBUB3</i>	E Q - C A V A S L C G I A V A L I D A S P - - V Q E K			
<i>scBUB3</i>	R F V E R E E L L N L K D T N L A P A S E S P R E K	250	260	270
<i>huBUB3</i>	E A A L A G G I K O N A E R R O N A A E P E N E L N			
<i>muBUB3</i>	E Y A S C E R E K E N A E S D O G E P A C A L E K N E S H Y			
<i>scBUB3</i>	F L Y L A P S D G I I S C V N L O T R K T N N A K F N I	290	300	310
<i>huBUB3</i>	E E A L G G P D A G P Y N E L D P A T N E L G E P C O E I R P E			
<i>muBUB3</i>	E F A L G G C S E D G I V A N E L D P S K N E L G A L I F V P S Y E			
<i>scBUB3</i>	D S I V Y K I A C E D N I L C A I S D D I F K T N A A I D O	310	320	330
<i>huBUB3</i>	E S I A A G G I G G I G G I G G I G G I G G I G G I G G I			
<i>muBUB3</i>	E S I A A G G I G G I G G I G G I G G I G G I G G I			
<i>scBUB3</i>	I I E L N A S S I I I I E D I N	340	350	
<i>huBUB3</i>	E E A G C I D E H R P O Y T D A Y E K P E S P C I			
<i>muBUB3</i>	E E E D C I E T A P R O P T E A V A G T G P K S I			

From Page No. _____

Overexpression of huBUB1, huBUB3, hsMAD2 and ttk in testis



This includes new TTK for exposure in high
 still needs to be put in notebook somewhere

To Page No. _____

Witnessed & Understood by me.

David Dahl

Date

Invented by

T. Spilley

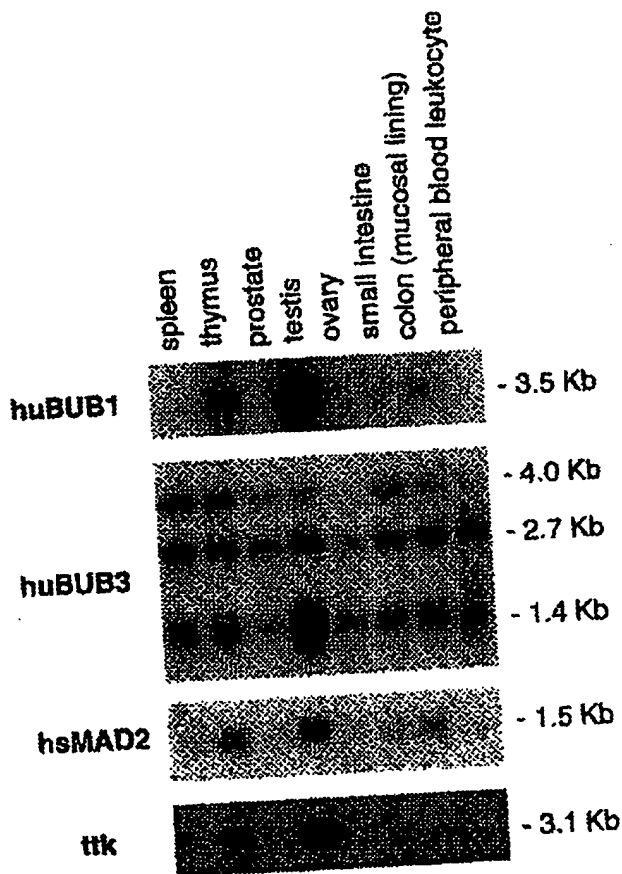
Date

Recorded by

4

From Page No. _____

Overexpression of huBUB1, huBUB3, hsMAD2 and ttk in testis



To Page No. _____

Witnessed & Understood by me,

[Signature]

Date

[Redacted]

Invented by

[Signature]

Recorded by

Date

[Redacted]

SEELEY
DECLARATION
EXHIBIT NO. 12

From Page

Addendum to Invention disclosure D-90-008

huBUB3, a novel human homolog of a yeast signaling gene

Todd Seeley

Prepared

A human cDNA sequence derived from overlapping public EST sequences (disclosure document) was used to design PCR primers (TWS95, TWS96). These primers were then successfully used to synthesize a PCR fragment roughly corresponding to the predicted huBUB3 open reading frame, using a human testis cDNA pool as template. The PCR fragment was cloned (291-2) and submitted for DNA sequence analysis. The fragment sequence was precisely as predicted from the EST analysis, with the exception of a single nucleotide (A predicted, C found; indicated with an asterisk* at pos. 667 in the following amended sequence). A reexamination of the ESTs overlapping this region showed that while A was originally retained from the EST alignments as the probable nucleotide at this position, C was predicted from EST H81956, and in other entries this position was recorded as ambiguous. Northern Blotting was used to examine mRNAs corresponding to the PCR fragment. mRNAs of three distinct lengths were observed; ~4.0, ~2.7 and the expected ~1.4 Kb. These results demonstrate that the earlier overlapping EST analysis indeed defines a contiguous mRNA transcript, which predicts the synthesis of a protein with homology to yeast BUB3. At this time, it is not clear if the ~4.0 and ~2.7 Kb mRNAs are derived from alternative splicing of the same gene as that which produces the expected ~1.4 Kb mRNA or if these mRNAs represent products of unique, related genes.

A human homolog of another yeast gene (scBUB2) known to act downstream in the same signaling pathway as BUB2 has been isolated as a transforming gene (the "tre" oncogene), using serial *in vitro* transfections of Ewing's sarcoma DNA. It is likely that human homologs of the other yeast genes in this pathway, including the huBUB3 sequence which follows, will exhibit a similar phenotype and may play important roles in carcinogenesis.

huBUB3

Sequence Range: 1 to 1225

```

                                primer TWS95>
                                -----60
                                30
GCGACCGAGC GATGTNNCG AGTAGTGGAA ACGETWCTT CTGAGGGGAG CCGAAGATGA
                                M>

----->
                                90
COGTTCTAA CGAGTCAAG CTGAACGAGC CACCCGAGGA TGGCATCTCC TCCGTGAAGT
T G S N E F K L N Q P P E D G I S S V N>

                                150
TCAGCCCCAA CACCTCCAG TCTCTGCTTG TCTCTCTCTG GCACAAGTCC GTGGCTCTCT
F S P N T S Q F L L V S S W D T S V R L>

                                210
ACGATGTGCC GGCAACTCC ATGGGGCTCA AGTACCAGCA CACCGGCGCC GTCTGGAAT
Y D V P A N S M R L K Y Q H T G A V L D>

```

Witnessed & Understood by me

David Auhl

Date

Invented by

T. Seeley

Date

Recorded by

li

TITLE

Patent

Project No. _____

Book No. _____

43

From Page 1

270 300
GCGCCTTCTA CGATCCAACG CATGCCCTGA GTGGAGGACT AGATCATCAA TTGAAAATGC
C A F Y D P T H A W S G G L D H Q L K M

330 360
ATGATTGAA CACTGATCAA GAAAATCTTG TTGGGACCCA TGATGCCCTT ATCAGATGTG
H D L N T D Q E N L V G T H D A P I R C

390 420
TTGATACTG TCCAGAAGTG AATGTGATGG TCACTGGAAG TTGGGATCAG ACAGTTAAAC
V E Y C P E V N V M V T G S W D Q T V K

450 480
TGTGGGATCC CAGAACTCCT TGTAACTCTG GGACCTTCTC TCAGCCTGAA AAGGTATATA
L W D P R T P C N A G T F S Q P E K V Y

510 540
CCCTCTCAGT GTCTGGAGAC CGGCTGATTG TGGGAACAGC AGGCCGCGA GTGTGGTGTG
T L S V S G D R L I V G T A G R R V L W

570 600
GGGACTTACG GAACATGGGT TACGTGCAGC AGGCCAGGGA GTCCAGCCTG AAATACAGA
W D L R N M G Y V Q Q R R E S S L K Y Q

630 660
CTGCTGCAT ACGAGCGTTC CCAACAAGC AGGGTATGT ATTAGCTCT ATTGAAGGCC
T R C I R A F P N K Q G Y V L S S I E G

* 690 720
GAGTGGCAGT TGATATTTCG GACCCAAGCC CTGAGGTACA GAAGAAGAAG TATGCCCTCA
R V A V E Y L D P S P E V Q K K K Y A E

750 780
AATGTCACAG ACTAAAGAA AATAATATG AGCAGATTTA CCCAGTCAAT GCCATTCTCT
K C H R L K E N N I E Q I Y P V N A I S

810 840
TTCACATAT CCACATACA TTGOCACAG GTGGTCTGA TGGCTTTGTA AATATTGGG
F H N I R N T P A T G G S D G F V N I W

870 900
ATOCATTTA CAAAAGCGA CTGTGCCAAT TCCATCGSTA CCCACAGAGC ATCGCATCAC
D P F N K K R L C Q F H R Y P T S I A S

930 960
TTGCCCTCAG TAATGATGGG ACTACGCTTG CAATAGCGTC ATCATATATG TATGAAATGG
L A F S N D G T T L A I A S S Y M Y E M

990 1020
ATGACACAGA ACATCCTGAA GATGCTATCT TCATTGCGCA AGTGACAGAT GCAGAAACAA
D D T E H P E D G I F I R Q V T D A E T

1050 1080
AAGCCAGTC ACCATGTACT TGACAAGATT TCATTACTT AAGTGCCATG TTGATGATA
K P K S P C T *
<primer TMS96

1110 1140
TAAACAATT CGTACTCCCC AATGGGGAT TTATTACTAT TAAAGAAAC AGCGAAATA
1170 1200
TTAATTTTA TATTATACA ACCTGAAAT AATGAAAAG AGGTTTTTGA ATTTTTTTT
TAAATAAAC ACCTTCTTAA GTGCA

2

Page No.

Witnessed & Understood by me.

David Dahl

Date

Invented by

T. Suley

Date

Recorded by

NB89480

043

2

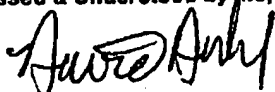
From Page No. _____

Human and murine homologs of *scBUB3*

scBUB3	M I V G B A K Y D E I I K S L
huBUB3
muBUB3
scBUB3 G T K F D I K N V D L L Q S
huBUB3
muBUB3
scBUB3	P - C N I N D L I Y V T Q Q L K V
huBUB3
muBUB3
scBUB3	I Q S F S P A T N G I C R I K Y G D D K
huBUB3
muBUB3
scBUB3	A G L E I N Y G D G V I A V K N L
huBUB3
muBUB3
scBUB3	T K Y K N D T N S S M N S Q W
huBUB3
muBUB3
scBUB3	F R P L C E D D G T I E G I D A L L K
huBUB3
muBUB3
scBUB3	B B A C F D Q Q D Y N S S
huBUB3
muBUB3
scBUB3	N L K D I N L A B S P R K
huBUB3
muBUB3
scBUB3	P L Y A I S C N L Q K A N E
huBUB3
muBUB3
scBUB3	D V K C D N I L T D D T F K T N A A I
huBUB3
muBUB3
scBUB3	T I L N A S S Y I P Y P C T
huBUB3
muBUB3

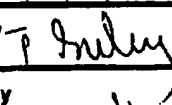
o Page No. _____

Witnessed & Understood by me,



Date

Invented by



Date

Recorded by

hcn3

Human and murine homologs of *scBUB3*

<i>scBUB3</i>	10	20	30
<i>huBUB3</i>	MQIVQHEA	KKYD	ISLIY-SKSL
<i>muBUB3</i>	MTIVQHEA	KKYD	ISLIY-SKSL
<i>scBUB3</i>	40	50	60
<i>huBUB3</i>	LTWV	KFDI	KNVDLLQS
<i>muBUB3</i>	LTWV	KFDI	KNVDLLQS
<i>scBUB3</i>	70	80	90
<i>huBUB3</i>	LP	LC	NIN
<i>muBUB3</i>	LP	LC	NIN
<i>scBUB3</i>	100	110	120
<i>huBUB3</i>	IGSPS	FCA	ITN
<i>muBUB3</i>	IGSPS	FCA	ITN
<i>scBUB3</i>	130	140	150
<i>huBUB3</i>	PEYA	WVGL	IEE
<i>muBUB3</i>	PEYA	WVGL	IEE
<i>scBUB3</i>	160	170	180
<i>huBUB3</i>	NTKVKN	ITN	SS
<i>muBUB3</i>	NTKVKN	ITN	SS
<i>scBUB3</i>	190	200	210
<i>huBUB3</i>	FR	PLCEDD	NGT
<i>muBUB3</i>	FR	PLCEDD	NGT
<i>scBUB3</i>	220	230	240
<i>huBUB3</i>	E	E	AC
<i>muBUB3</i>	E	E	AC
<i>scBUB3</i>	250	260	270
<i>huBUB3</i>	FLY	A	ES
<i>muBUB3</i>	FLY	A	ES
<i>scBUB3</i>	280	290	300
<i>huBUB3</i>	FLY	A	ES
<i>muBUB3</i>	FLY	A	ES
<i>scBUB3</i>	310	320	330
<i>huBUB3</i>	D	VVK	IC
<i>muBUB3</i>	D	VVK	IC
<i>scBUB3</i>	340	350	
<i>huBUB3</i>	TI	ELN	ASS
<i>muBUB3</i>	TI	ELN	ASS

Andre Ardy

T. Miley

MDIVQIEQAKFDYISDFELIRSKSL
MTCGSLNPLKHNQPCEDDGLISVAVKSPNLSQD
MTCGSLNPLKHNQPCEDDGLISVAVKSPNLSQD

40 50 60
STTAYDGLITVKKFDIAKKNVDELIQSIRK
AVESLYDDESLRCLDVAFAKQPHLPSYQ
EVSISANDTSEVSLRCLDVAFAKQPHLPSYQ

70 80 90
HPCLCCNFIQNDILQIYVTVJGETILKV
HPCALVADCAVADTTHAVVSGTSLDILQKCH
HPCALVADCAVADTTHAVVSGTSLDILQKCH

100 110 120
DGLGSPSTQAITNNEENIGICKIKYGGDK
DGLGSPSTQAITNNEENIGICKIKYGGDK
DGLGSPSTQAITNNEENIGICKIKYGGDK

130 140 150
LIAAEDWGLIEVITNYGGGVIAVKNLIS
LIAAEDWGLIEVITNYGGGVIAVKNLIS
LIAAEDWGLIEVITNYGGGVIAVKNLIS

160 170 180
NNTKYVKNIFPMDTNSSRLTYGMNNSQWQW
NNTKYVKNIFPMDTNSSRLTYGMNNSQWQW
NNTKYVKNIFPMDTNSSRLTYGMNNSQWQW

190 200 210
FRFPICEDDNGTIEGGRVQLRQVAILLK
FRFPICEDDNGTIEGGRVQLRQVAILLK
FRFPICEDDNGTIEGGRVQLRQVAILLK

220 230 240
FEFCFACSSIDRFFFDQGDYNSSS
FEFCFACSSIDRFFFDQGDYNSSS
FEFCFACSSIDRFFFDQGDYNSSS

250 260 270
RFAFRDTHNLKDTNLAIVYNSDESKFA
RFAFRDTHNLKDTNLAIVYNSDESKFA
RFAFRDTHNLKDTNLAIVYNSDESKFA

280 290 300
FLYGAEDDGLISVAVKSPNLSQD
FLYGAEDDGLISVAVKSPNLSQD
FLYGAEDDGLISVAVKSPNLSQD

310 320 330
DGVVVKIACSDNILEVSLDDETKTNAAIQD
DGVVVKIACSDNILEVSLDDETKTNAAIQD
DGVVVKIACSDNILEVSLDDETKTNAAIQD

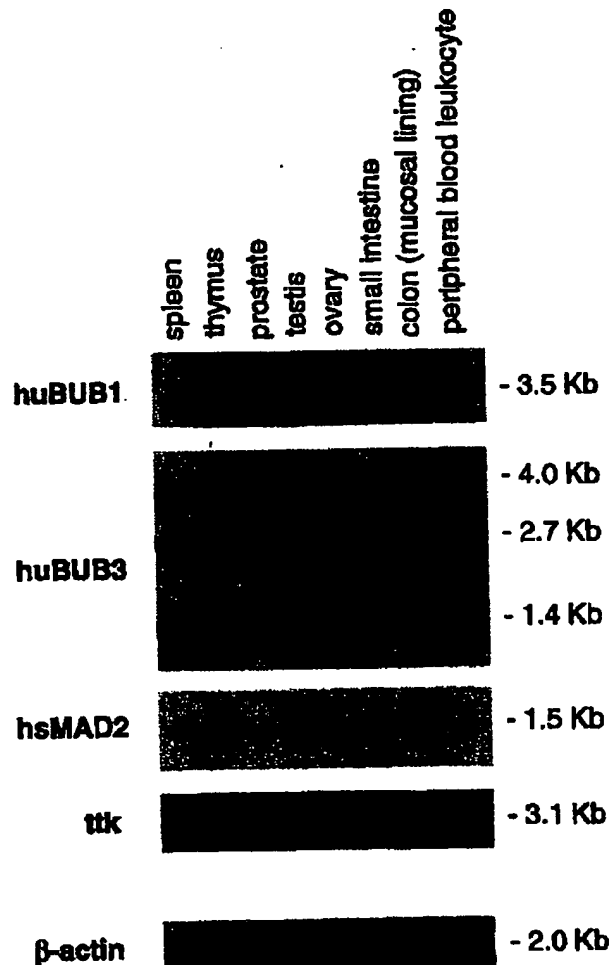
340 350 360
IILNASSHIIIFDIN
IILNASSHIIIFDIN
IILNASSHIIIFDIN

SEELEY
DECLARATION
EXHIBIT NO. 13

From Page No. _____

Prepared data for lab mty. Mike
never showed.

Overexpression of huBUB1, huBUB3, hsMAD2 and ttk in testis



Witnessed & Understood by me,

David Dahl

Date

[REDACTED]

Invented by

T. Huley

Date

[REDACTED]

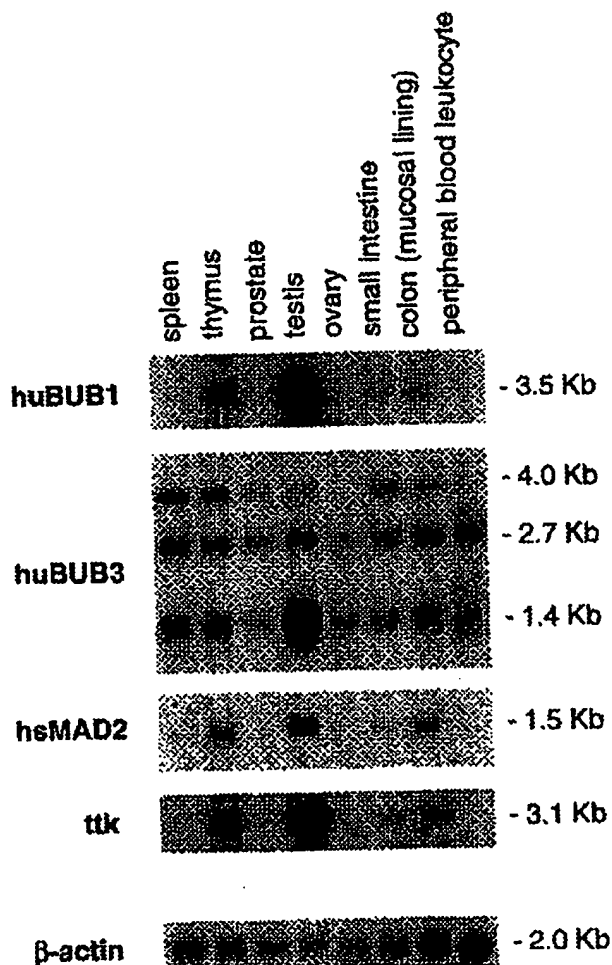
Recorded by

h

From Page No. _____

Prepared data for Lab intg. Mike
never showed.

Overexpression of huBUB1, huBUB3, hsMAD2 and ttk in testis



To Page No. _____

Witnessed & Understood by me,

Date

[REDACTED]

Invented by

T. Miller

Date

[REDACTED]

Recorded by

4

SEELEY
DECLARATION
EXHIBIT NO. 14

From Page No.____

Spoke to Mark Selby about trying
in situ stain. He strongly recommended
Flag-tagging. Here is his NS-2
triple Flag-tagged protein.

This is a 3 tagged
 sequence - I
 will do 5 using
 2 long steps.
 Using codon and
 freq. tables introduced
 a genome. Introduced
 lots of new bp changes
 to allow annealing
 of low salt buffer
 with overhangs
 Flanking

2822 ILeGlnSerLeuLysAspAspProSerGlnSerAlaAsnLeuLeuAlaGluAlaLysLys
ATCCAAGTTTAAAGATGACCCAGCCAAAGCGCTAACTTTTACGCAAGACTTAAAG
TAGGTTTCAAATTTTCTACTGGGTTCCGGTTTCGGGATTGGAAGATCGTCTTCGATTTTC

2830 AHA3, 2852 SCO473.

2882 LeuAsnAspAlaGlnAlaProLysValAspAlaAsnSerGlyGlyGlyGlySerAlaAsn
CTAATCATGCTCAGGCGCCGAAGTAGACGGCGAATTCGGGAGCGGGGGGCTCAGCCAC
GATTTACTACGAGTCGCGGGCTTTCATCTGCGCTTAAAGGCTCCGCCCCGAGTCGGTTC

2896 KASI NARI, 2914 ECORI, 2931 ESP1.

2942 A1aGluAspValValCysCysSerMetSerTyrSerTrpThrGlyAspTyrLysAspAsp
GCGGAGGATGTGCTGTCTGTCTCAATGCTTACTCTGGACGGGTCACTCAAGGACGNT
CGCTCTCTACAGCACACGACGAGTTACAGATGAGAACTGGCCACTGATGTCTCTCTA

2981 AGE1.

3002 AspLysGlyAspTyrLysAspAspAspLysGlyAspTyrLysAspAspAspLysAsn
GACAAAGGACACTACAAGCAGCTGCACAAGGAGACTACAGGACGGTGCACAAATAGGCT
CTGTTTCTCTGATGTTCTGCTACCTGTTCTCTGATGTTCTGCTACTGTTTATCCTA

3058 BAHMI.

3062 CCTCTAGAGTGGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGT
GGAGATCTCTAGCTGGACATCCGTACGTTGGAACCGTGACGGGCAGCAAAATGTTGCAGCA

3064 XBAI, 3070 SALI, 3075 SSE83871, 3076 PSTI, 3082 SPHI, 3088 HINDI.

K3 A AGCTT
 A CCGGT
 A
 Single cuts
 for ACG3.1/
 BUB3 sense

DUB3-FLAG Translated Sequence

Sequence Range: 1 to 93

H3

AGCTTATGGA CTACAAGGAT GACGACAAGG GAGACTACAA AGACGATGAC AAAGGGGGATT

M D Y K D D D K G D Y K D D D K G D


FLAS 1

ACAAGGACGA CGATAAGGGC GGAGGCGGGG CCA

Y K D D D K G G G G G X

30 60 90

Asp 2

ordered ³  linker
TWS 134 sense 5' phos
TWS 135 2 sense "

Witnessed & Understood by me.

Date**Invented by**

Date _____

Recorded by

Page No

Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 14

087

From Page No.____

Spoke to Mark Selby about trying
in situ stain. He strongly recommended
Flag tagging. Here is his MS-2
Antibody Flag-tagged protein.

this to a 3 tagged
 sequence - I
 will do 5' using
 a long oligo.
 Using cotton
 thread. Introduced
 a few 22. Introduced
 a new bp changes
 both new annealing
 to allow annealing
 of low salt
 with 10%
 10%

[illegible]

HB A. AGCTT
HB A. AGGT
HB A
Single cuts
for PUB3.1/
PUB3 sense

Wt3-FLAG Translated Sequence

Sequence Range: 1 to 93

H2

30 60

AGCTTATGGA CTACAAGGAT GACGACAAGG GAGACTACAA AGAAGATGAC AAAGGCGATT

M D Y K D D D R G D Y K D D D K G D

FLAS 1

90

ACAAGGACGA CGATAAGGCG GAGGCGGCGG GGA

Y K D D D K G G G G G D

3 linker

ordered

TWS 134 sense

TWS 135 2 sense

5' phos

Witnessed & Understood by me.

Date:

Invented by

Discussion

Recorded by _____

From Page No. _____

Cont.

Prep gel -

- 1 " "
- 2 291 Pin / H3
- 3 " "
- 4 " "
- 5 291 Pin
- 6 291 H3
- 7 lig oligos.

Results: great!!

Cut out Pin/H3 291,

and oligo ladder

from ~600 - 2kb

from gel slices @ -20°C.

Qiaquick gel extracted DNA's;

2 cols ea.

elute ea 30x vol = 60 total.

Digest oligo ladder

134/135	562
DW	114
10x B	20
PINAT	5
H3	5
	<u>200</u>

dig chi, start 12:45

Run Qiaquick nucleotide removal

ARSP 30A

dix 1:20 for rapid lig

291	A/H	1A
1-20	134/135	3A
	5x DNA	12
2x lig		5A
		1A

inc 7.0.5 hr;
put to -20°C to
stop tomorrow.

To Page No. _____

Witnessed & Understood by me,

David Duhl

Date

Invented by T Suley

Date

Recorded by u

From Page No.____

Cell	Assay	pH	280	260/280	µg/ml OD	µmol stock	µmol/ml OD	µmol/stock	ml /100 µmol	cell 200m/ul
TWS134	BUB3-FLAG	93	0.060	0.092	27.6	828	899	27	3.71	1.9
TWS135	BUB3-FLAG	93	0.085	0.073	34.2	1625	1151	55	1.83	2.7

obito

took on's -

annealing

$\text{DAS } \begin{array}{r} 121 \\ 35 \end{array} \quad \begin{array}{l} 15 \text{ ml} = 124 \text{ ml} \\ 9.3 = 11.9 \text{ ml} \\ \text{DW} = 62.5 \text{ g} \end{array}$
 $\rightarrow 8 \times \text{digest} = 12.6 \text{ g}$

found -
in -20°C
from early
progeni
work

heat $94^{\circ} 2'$
 $68^{\circ} 2'$
 $\downarrow 5'$
 $4^{\circ} C$

put to -20°C

Digest 291-2 = BuB3 sense (CR3.)

Pin A1/H3. Pin A1 is immed @ 5' end of BUB3 ORF.

~~ARE~~ Procedures
DNA 10
PW 70

And this (1 enzo)

DNA	11
DW	6
10 x	11
em 4	1
	10

(AgI)

Wk B	10
P in A1	5
H3	5
	<u>100</u>

das ist

diğer 1hr.

Polymerize 134/135 Anneal tags

0	0	100 µl
5x	model	20 µl
2x	lig	120 µl
lig		<u>10 µl</u>

0.5 hr rapid leg; then
Quincke Per

To Page No.

Witnessed & Understood by me

Read & Understood by me.

Date**Invented by**

Recorded by

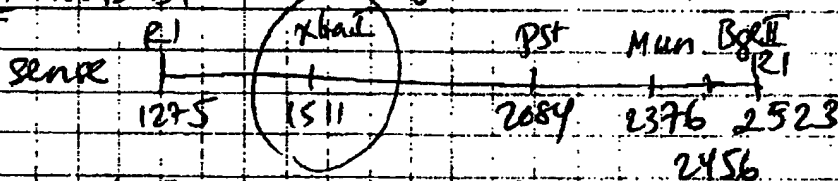
Date

From Page No. 45

San Diaprep 815 on on's 8 la from
lig 321 = BUB1 - pcr3.1 ~1.3 kb frag

lig 322 = BUB3-flag tag

321 linBUB1: 1248 nt frag R1 - R1



decided to use XbaI.

lin 5.0 if no insert (-pcr3.1)

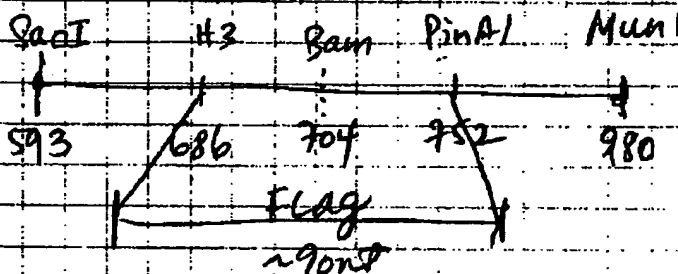
1012 frag if sense

236 frag if antisense

6.2 kb total

322

BUB3-FLAG



look for loss of BamHI; look for Sac/Mun = 411

321 Xba

	1x	9x
DNA	3	85.5
DW	9.5	13.5
10x H	1.5	9
enz	1	

322 Bam

	1x	10x
DNA	3	95
DW	9.5	15
10x B	1.5	10
enz	1	

322 Sac/Mun

	1x	10x
DNA	3	90
DW	9	15
10x "L"	1.5	7.5
enz 1	.75	7.5
enz 2	.75	7.5

322 San 291 Controls in digests

Witnessed & Understood by me,

David Dull

Date

Invented by

T. Dull

Recorded by

u

Date

To Page No. _____

From Page No. _____

33

dig 2nd; then ran gels

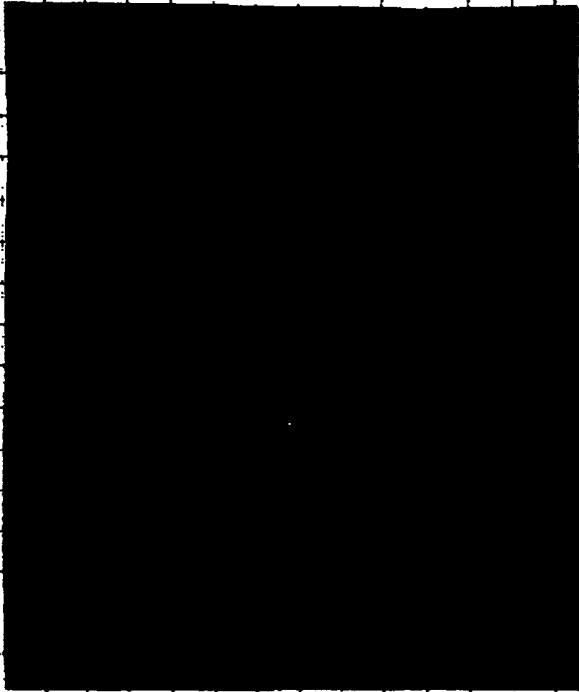
321 Xba

322 Bam

1/2 gel

322 Sac/Mun 4/1 gel

321 Xba



322 S/M



322 bam

Results: 321 (BUB-PCR3.1) None?! all look like 5.0 kb = vector, no insert, except #6 which looks like either Δ or Xba no cut.

322 (BUB Flag). Looks like I oughta checked Bam sites 1st; all 8 look diff from vector. Clearly s/m all diff from vector; doesn't look like multi-inserts.

Sent 322-1,2,3 to Fastrak; check C pT7 primers.

To Page No. _____

Witnessed & Understood by me.

Date

Invented by

T. Luley

Recorded by

1

Date

Project No. _____

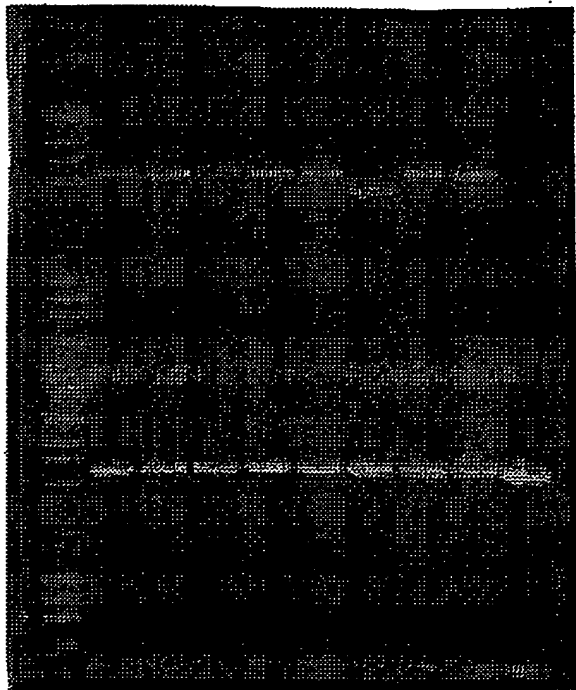
Book No. _____

TITLE BUB1 (PCR3.1) + BUB3 Flag tagFrom Page No. 133dig 2nd; then ran gels

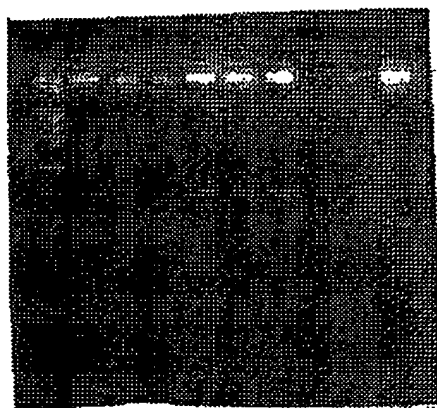
321 Xba
322 Bam | 1 gel

322 Sac / Mun 4 gels.

321 Xba



322 S/M



X

322 bam

Results: 321 (BUB PCR3.1). None?! all look like 5' p. 6b = Vector, no insert, except #6 which looks like either Δ or Xba no cut.

322 (BUB Flag). Looks like I oughta checked Bam sites 1st; all 8 look diff from Vector. Clearly S/M all diff. from Vector; doesn't look like multi-inserts.

Sent 322-1,2,3 to Fastrak; check 2 pT7 primers.

To Page No. _____

Witnessed & Understood by me.

[Signature]

Date

[Redacted]

Invented by

[Signature]

Recorded by

7

Date

[Redacted]

TII

TITLE

PCR 3.1 t6c-1; BUB1 (BUB3)

Project No. _____

B688 No. _____

149

From Page No. _____

cont

	1x	10x
DNA	B	
DM	9.5	9.5
10x	1.5	1.5
enz	1	10

Xba = "H" buffer
 Bam = B
 EcoRV = B

dig 1 hr; run in gel.
 321 Xba

Results:

321: all same except #5.
 #5 looks like 5.0 kb vector; so all same orient?

A short Xba to Xba would mean X sense; this poss Xba doesn't cut and this is just linear.

Need to analyze these with more enz.

329 Bam

RV 329.

lg scale preps

329. 2 orientations.

Picked 321-1, 329-1, 329-2 to do lg scale 100 ml cultures. (6/18 submit to Fastrak)

Also; today ran lg scale preps on 322-1 (BUB3-FLAG) and 321-4 (old 321 = PCR3.1) left @ -20°C as dry pellets.

321. Could I have cloned a different BUB1 frag (e.g. no Xba) than I thought?

To Page No. _____

Witnessed & Understood by me,

Hawco Dahl

Date

Invented by

T Hulley

Date

Recorded by

9

TITLE

PCR 3.1 t6c-1; BUB1 & BUB3

Project No.

Page No.

149

From Page No.

[Redacted]

cont

DNA

1x

10x

DW

9.5

9.5

10x

1.5

1.5

enz

1

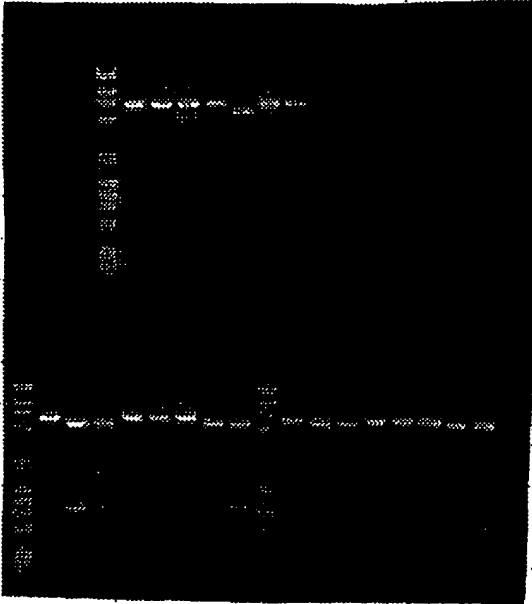
10

Xba = "H" buffer

Bam = B

EcoRV = R

dig 1 hr; run 1% gel
321 Xba



Results:

321: all same except #5
#5 looks like 5.0 kb
vector; so all
same orient?

A short Xba to Xba would
mean a sense; tho.
pass Xba doesn't cut
and this is just linear.

Need to analyze these
with more enz...

329 Bam

RV

329

lg scale preps

329. 2 orientations.

Picked 321-1, 329-1, 329-2 to do lg scale
100 ml cultures. (6/18. Submit to Fasttrak)

Also; today ran lg scale preps on 322-1
(BUB3-frag) and 321-4 (old 321 = PCR3.1)
left @ -20°C as dry pellets.

321. Could I have cloned a different BUB1
frag (e.g. no Xba) than I thought?

To Page No.

Witnessed & Understood by me:

Haver Dahl

Date

Invented by

T. Suley

Date

Recorded by

4

SEELEY
DECLARATION
EXHIBIT NO. 15

From Page No. _____

I ordered BMB rabbit retic. lysate and
"TA-Capscribe" to make in vitro tx'd
labelled proteins. Idea is to see if

Flag BUB3 can co-IP other tx'd proteins

Spoke to Mercier; who does "TNT" from Promega;
using pools of 20 plasmids at a time
and decided to go ahead and do it her
way. They transcribe/translate in a
single reaction with Promega kit; 100 ng/ μ
pool of 12 plasmids;

1 μ l DNA + 4 μ l TNT mix.

Made dilutions of following plasmids.

	Desig	Cont	Conc	Vol/cont
1	283-1	MAD2 sense	2800 ng/ μ l	0.71
2	283-5	MAD2 α sense	3030	0.65
3	291-2	BUB3 sense	1800	1.06
4	291-5	BUB3 α sense	1210	1.65
5	336-38	PCR2.1 sense & BUB1	1430	1.39
6	337-50	PCR3.1 sense 4	170	11.8
7	337-61	" "	410	4.87
8	422-1	Flag BUB3	650	3.07
9	PCR3.1	(Vector)	570	3.5

To Page No. _____

Witnessed & Understood by me.

Hawco Buhl

Date

Invented by

T. J. Juley

Date

Recorded by

n

From Page No. _____

Marc's instructions.

1x DNA

4x Int. mix.

For 32 samples	Microsome DNA	0.24 ml/sample 1.2 ml/sample
Reticulocyte	80 ml	80 ml
Reaction Buffer	6.4	6.4
SP6	3.2	3.2
AA Minus Met	3.2	3.2
RNA sin	3.2	3.2
³⁵ S-AA	12.8	12.8
<Microsome>		7.68
Nuclease-free H ₂ O	(19.2) (14.2)	(11.52) (6.52)
³⁵ S-S	(15) (14.2)	(15) (14.2)

She says I can use all her reagents
and her bench for ³⁵S work since
she's doing almost all tissue cult work.
Was recall vols based on above ratios

	1x	11x
Reticulo	2.8	27.5
React	0.2	2.2
T7	0.1	1.1
AA - met	0.1	1.1
RNA sin	0.1	1.1
³⁵ S - met	0.4	4.4
bw	0.6	6.6

Start label 10:07

Stop running gel 11:37
= 90 min.

gel 10-20% Novex

van all of ea sample

plus 5ul Biolol

"Blue Juice"

And can get Biorad Broad range markers #161-0318
gel: 220V/hr; fix Coomassie destain, tris gel
dry 1h 80°C; put Biomax film R₁ on.

To Page No. _____

Witnessed & Understood by me,

David Huh

Date

Invented by

T Luley

Date

Recorded by

C1

SEELEY
DECLARATION
EXHIBIT NO. 16

From Page No. _____

18 flag-BUB3 pulls down BUB1/MAD2, try
 serial PCR deletion mapping to define
 interacting regions. makes no sense to
 clone if can be avoided. Kit says
 eg. A helps B. efficiency.

ATZ met ATZ PA(n)
 templ.

→ Could use this to map or HA tag other
 frags; this probably ought to keep ~~frags~~
 relatively small to avoid heterogeneity
 in PCR prod. form.

The results: Great! Bands!!

Calibrated Molecular Weights of Prestained
 SDS-PAGE Standards*

PROTEIN	HIGH RANGE	LOW RANGE	BROAD RANGE
Control #	871605	874177	872805A
Myosin	205,000	—	208,000
β -galactosidase	116,000	—	119,000
Phosphorylase B	—	107,000	—
BSA	85,000	76,000	79,500
Ovalbumin	47,000	52,000	49,500
Carbonic anhydrase	—	36,000	34,800
Soybean trypsin inhibitor	—	27,200	28,300
Lysozyme	—	19,000	20,400
Aprotinin	—	—	7,200

* Molecular weights are of representative lots; actual weights may vary. The
 lot-specific molecular weights are included with every vial.

Myosin
 β -galactosidase
 Bovine serum albumin
 Ovalbumin
 Carbonic anhydrase
 Soybean trypsin inhibitor
 Lysozyme
 Aprotinin



Calc. sizes based on
 dil. molarity.

MAD2 27K
 BUB3 42K
 FLAG BUB3 46K
 BUB1 110K

Witnessed & Understood by me,

David A. Stahl

Date

Invented by

T. Indley

Recorded by

Date

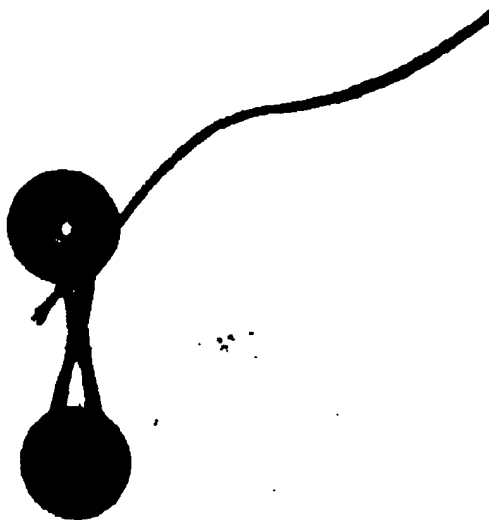
Serial No. 10/084,700

Filed 02/27/2002

SEELEY DECLARATION

EXHIBIT NO. 16

From

NOTEBOOK # 10148PAGE # 72DATE: [REDACTED]NAME: Todd Seeley

NOTES:

In vitro translationreaction products³⁵S-label

Witnessed & Understood by me

David D. Dahl

Date

[REDACTED]

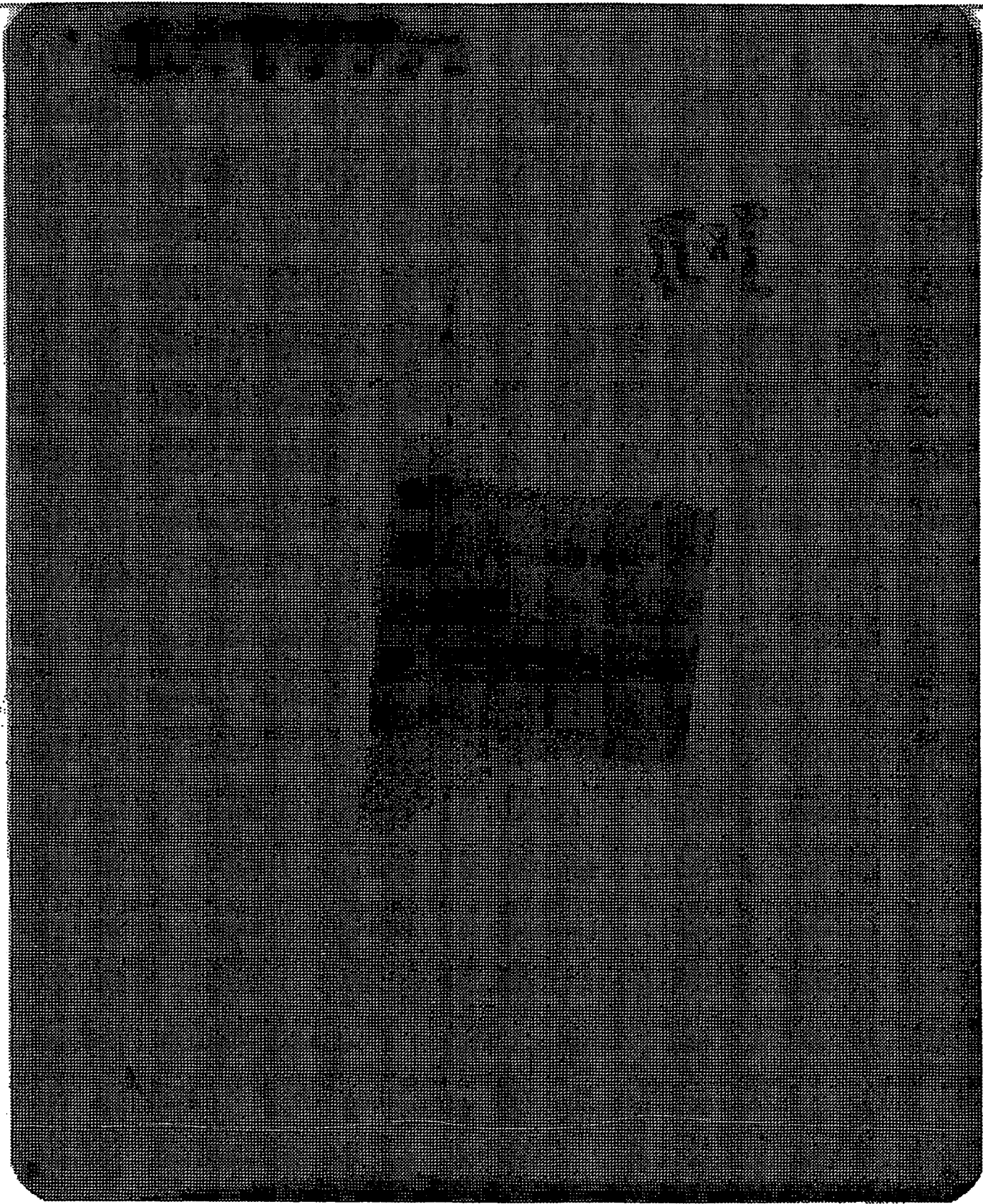
Invented by

Recorded by

T. Seeley
h

Date

[REDACTED]



SEELEY
DECLARATION
EXHIBIT NO. 17

From Page No. _____

Use "Anti Flag M2 Affinity gel"
(Kodak # IB 3020)
to pull down Flag BUB3.

test specificity and anti Ab q'd.
template

Flag Ab	BUB	Flag BUB
0 μ l	Rxn #1	#1
1 μ l	#2	#2
2 μ l	#3	#3
4 μ l	#4	#4
6 μ l	#5	#10

DNA + labeling mix

inc 2 Ab

separate in cfg

run gel of pellet, supernatant

BNA's "BUB" BUB 3 sense
"BUB" Flag BUB3

see pg 62.

label mix

	11 μ l
Refric	27.5
Tnt React	2.2
T7	1.1
AB-met	1.1
RNasin	1.1
35S met	4.4
DW	6.6

Start label 11:45 30°C
12:15 add Ab to label mix

Affinity gel washed 1x TNE
50 mM Tris 7.5 (10 sec spin)
2 mM EDTA
100 mM NaCl.

Binding Reaction:

4 μ l label mix
16 μ l Abs in TNE.
20.

2:00 begin washes TNE
did 2 each.

gel 1. M SP SP ... SP
#1 #2 #5

gel 2 MM SP SP ...
#6 #7

20 μ l label
+ 5 μ l DNA

20 μ l label
+ 5 μ l DNA

0 2 4 6

IP reactions
Flag agarose

Results: gels as before
good, not perfect. pnt'n c/o Ab.
but Flag was good.

Witnessed & Understood by me,

David Dahl

Date

Invented by

T. Enly

Date

Recorded by

u

Project No. _____

Tnt Flag Ab test

10

Frc



NOTEBOOK # 1048

PAGE # 162

DATE: _____

NAME: Todd Seely

NOTES:

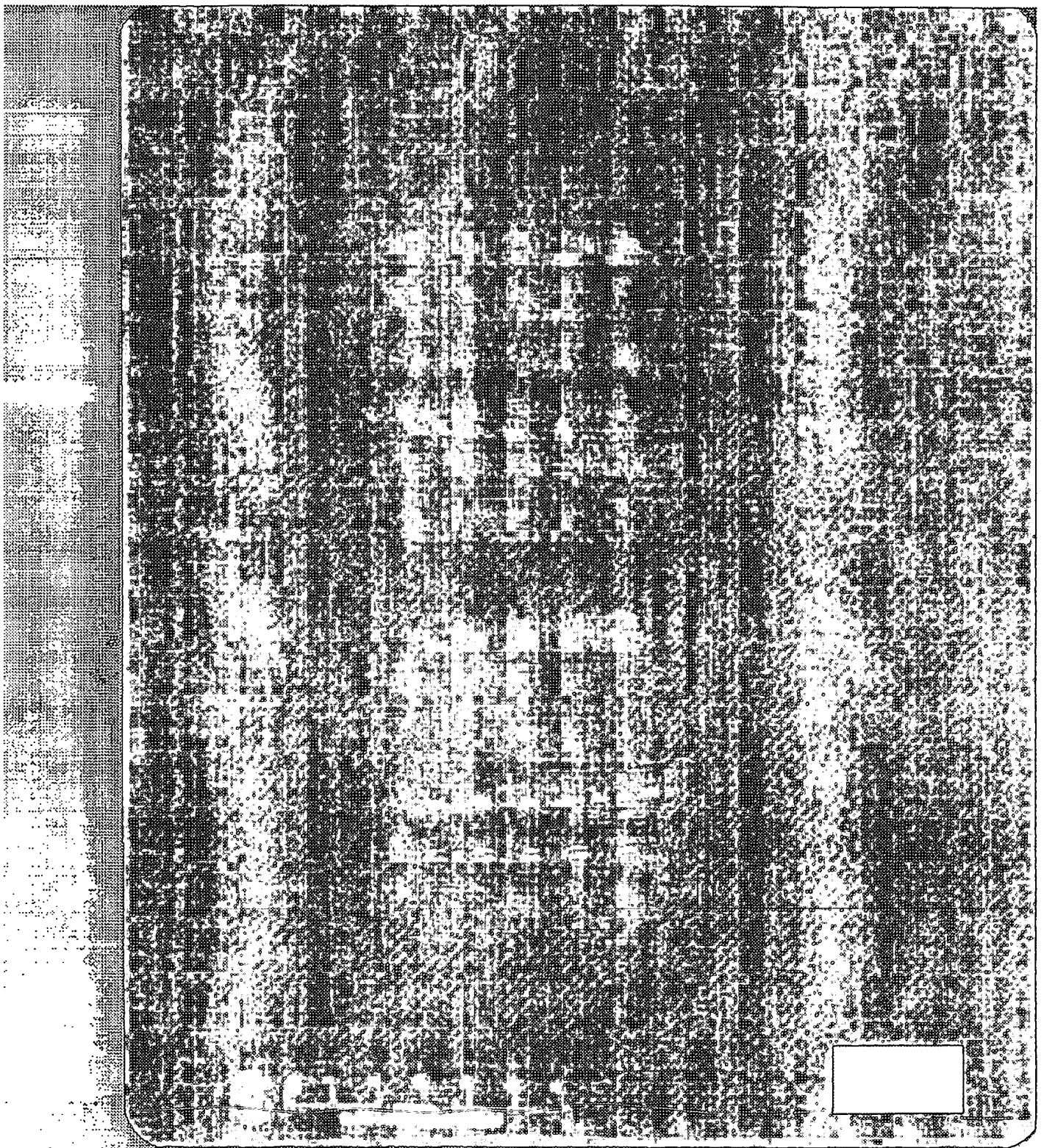
Tnt reaction
BUB3, BUB3 Flag pntn c & Flag Abs.

Wtr

David Buhl

Recorded by

T Seely



SEELEY
DECLARATION
EXHIBIT NO. 18

From Page No. _____

cont

Ln	Contents	Vol DNA / labeling
1	DNA ①	
2	MAD2 sense	0.2 + 0.8 DW
3	BUB3 sense	0.3 + 0.7
4	Flag BUB3	0.3 + 0.7
5	⑤	
6	BUB1's (see pg 62)	0.5 + 0.5 2x
7	⑦	
8	1, 3, 5	
9	1, 3, 6	MAD2, BUB3, BUB1 } 0.2 + 0.3 + 0.5
10	1, 3, 7	
11	1, 3, 5	
12	1, 3, 6	MAD2, Flag BUB3, BUB1 } 0.2 + 0.3 + 0.5
	1, 3, 7	

mix	Vol	13.5x
latic	2.5	33.75
React	0.2	2.7
TA	0.1	1.35
Q9-met	0.1	1.35
RNAsin	0.1	1.35
SS met	0.4	5.4
DW	0.6	8.1

Full DNA +

4x Tut mix

inc 30°C start 12:15

8 µl Ab washed TNE 1ml
 Spin 10" Resp 202.57 TNEs
 WAE 157 / TNE (= 67 AB + 97 TNEs equiv)
 AB added @ 1:40 p. inc RT

2:20p Spin, take 10 µl supe

wash 1x 200x TNEs

2x 200x TNE

resp pellet 10x 2x SB, add 10x 2x SB to supe

load all.

gel 10-20% as before; 220x.

gel 1: M S P SP SP
 1 2 6
 2: M M SP SP ...
 7 8

gel dried
 1h 80°C;
 fit over
 wkend

To Page No. _____

Witnessed & Understood by me,

David Ruhl

Date

Invented by

Tchuley

Recorded by

4 J

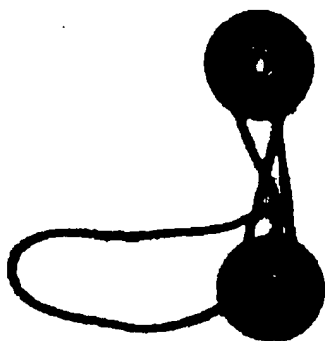
Date

Project No. _____

Mt CoIP.

11C

Fro



NOTEBOOK # 10148

PAGE # 110

DATE: [REDACTED]

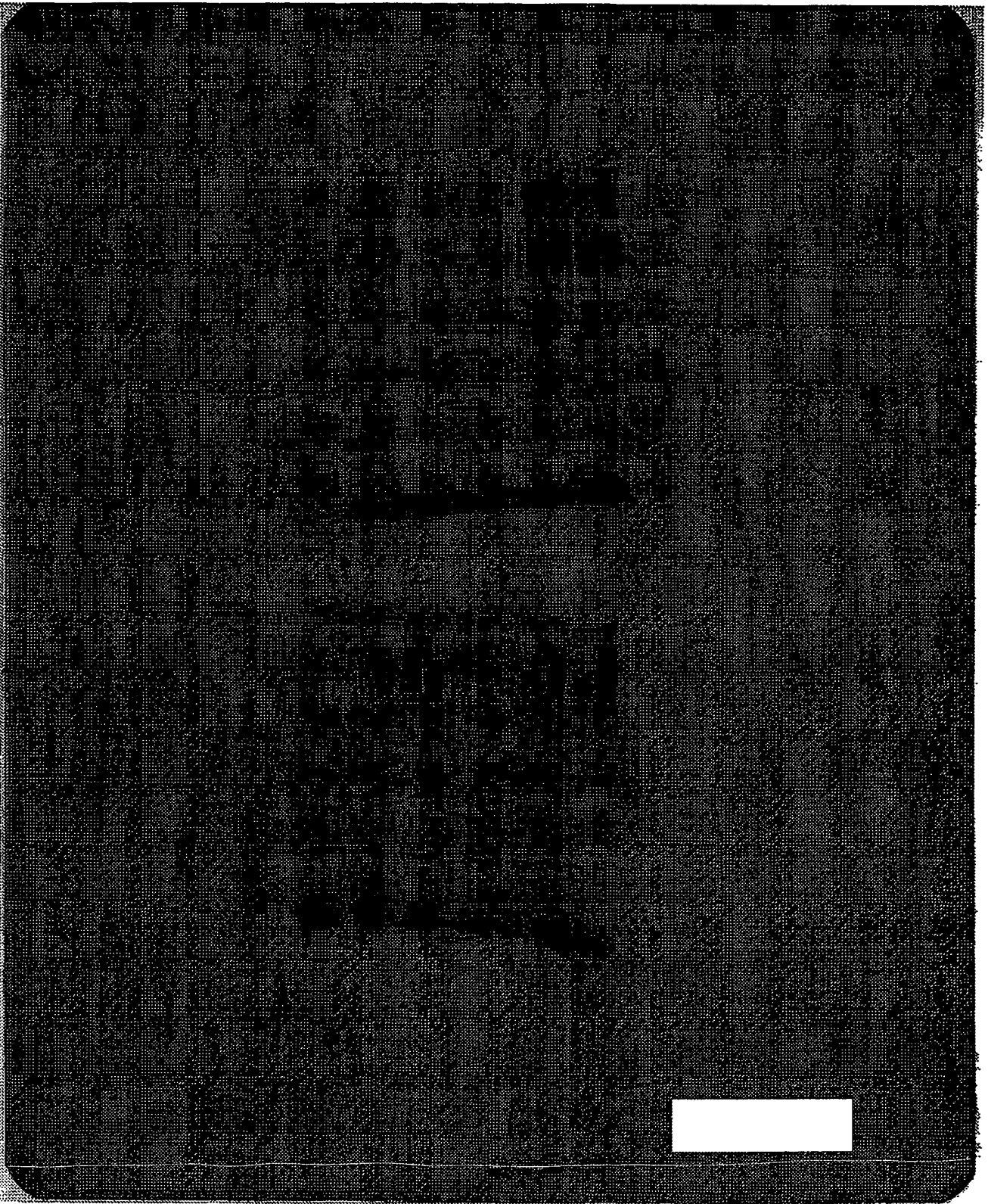
NAME: Todd Sealey

NOTES: 2 films from CoIP
Experiment in vitro Transl. expt.

Wit

Dave Dull

[REDACTED] Recorded by

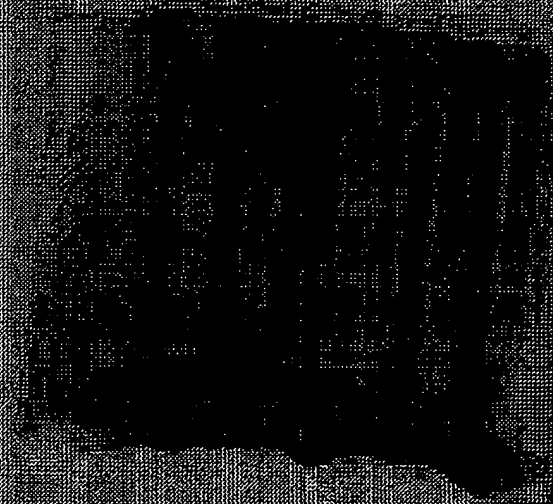
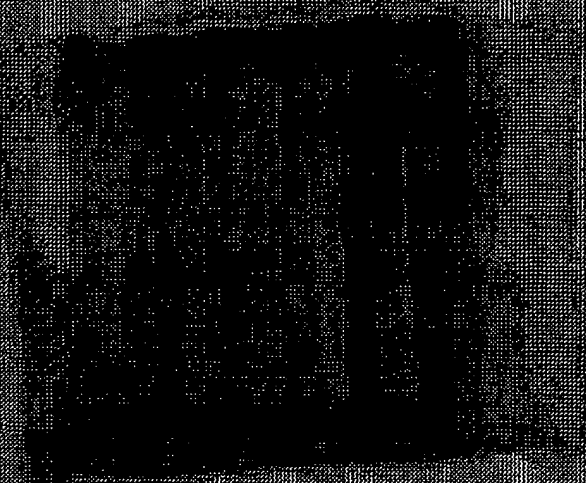


NB10148

0110a

3

RECEIVED



over wind
off



From Page No. _____

Results: Stephanie in Q2-01 put film. She uses other Biomax opt. for S35; much cleaner background. I should order some.

In the CoIP's, it's clear Flag BUB3 is enriched in pellet fraction; while BUB3 (noFlag) is depleted. This is same as previous result. It is also clear that singly, MAD2 and BUB1 are also depleted from pellet fraction. In mixed translations, MAD2 and BUB1 are also depleted if co-translated with BUB3 (noFlag). However, it looks as though BUB1 may ever so slightly be enriched in pellet fraction in Flag BUB3 co-translation reactions vs noFlag BUB3 pellets.

Clearly, pellets include protein which is not requiring antibody; and TNEs and extra pellet washes are not helping.

Need to lower background ppt'n Ab independent to see if BUB1 really is forming a complex with Flag BUB3.

It occurs to me that this Ab-independent ppt'n is aggregated material and can likely be removed by spinning Tnt reactions to remove insoluble labelled proteins prior to Ab incubations. This stuff must be heavily aggregated to come down in 10 sec spin.

To do - try clarifying reactions eg 1 min spin prior to Ab incubations.

To Page No. _____

Witnessed & Understood by me,

[Signature]

Date

Invented by

T. Suley

Date

Recorded by

cc

SEELEY
DECLARATION
EXHIBIT NO. 19

From Page No.

In last expt; noted 338-2 is poorly expressed.
noted PCR2.1 has 2 ATGs before BUB3 5' is
sequenced.

No ATGs exist in PCR3.1 T7 region;
this explains lousy data. Skip 338-2
till I decide what to do about this.

Repeat prev. Tnt expt; use only BUB1 (337.50);
BUB3 flag/no flag and new MAD2 error free
(344-6)
and adjust DNA ratios to improve data.

BUB3 0.3 μ l
BUB3 Flag 0.5 μ l
MAD2 0.4
337.50 0.15

decided to check BUB3/BUB3 Flag association
(\equiv BUB3 Self-association)

	14	50
Reac	2.5	12.5
77	12	1.0
aq-met	11	0.5
RNA sin	11	0.5
35S met	14	2.0
DW	10.5	2.5 0.15
#6 (337.50)	10	2.75 0.15
#10 (344-6)	14	2.0

4 tubes.

	NO BUB	BUB3	BUB3 Flag	BUB3 + BUB3 Flag
DW	1.0	0.3 +0.7 DW (#3)	0.5 +0.7 DW (#8)	0.15 +0.3 +0.2 DW (#3+8)

12 DNA + 4 μ l mix
Label start 11:20
12:15 wash (30s) imp
TNE
Resp 30s TNEs
12:20 9 TNEs to Tnt,
Spin 30", sup
to new tube.
add 6 μ l to
12:30 inc RT
1:40 stop; spin
15"
5 μ l sup to
new tube;

To Page No.

Witnessed & Understood by me.

David Auhl

Date

Invented by

T. Suley

Date

Recorded by

1

From Page No. 151

Supr / pellet fractionation

from p151:

Spin binding rxns 15"

Put 5% Supr to new tube

Wash pellet 1x 1ml TNE
2x 2ml TNE

10% SB to Supr

10% SB to pellet.

loaded 10% Supr + SB = 6.7% nominal
loaded all of pelletResults Fantastic! Clearest result yet.BUB1 / BUB3 clearly associate
Flag

BUB3 / BUB3 Flag do not.

MAD2 / BUB3 Flag do not.

Note 337-5/ BUB1 has a mutation in a
protein binding domain still.

To Page No. _____

Witnessed & Understood by me,

David Hall

Date

Invented by

G. Luby

Date

Recorded by

11

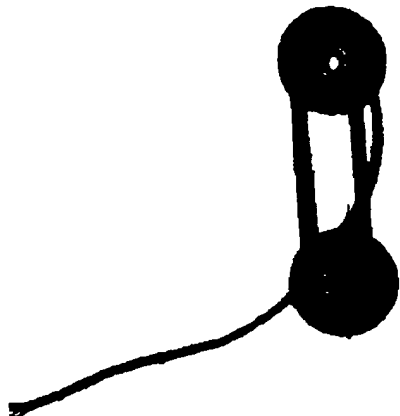
|||

Project No. _____

TII

153

Fri



NOTEBOOK # 10148

PAGE # 153

DATE: [REDACTED]

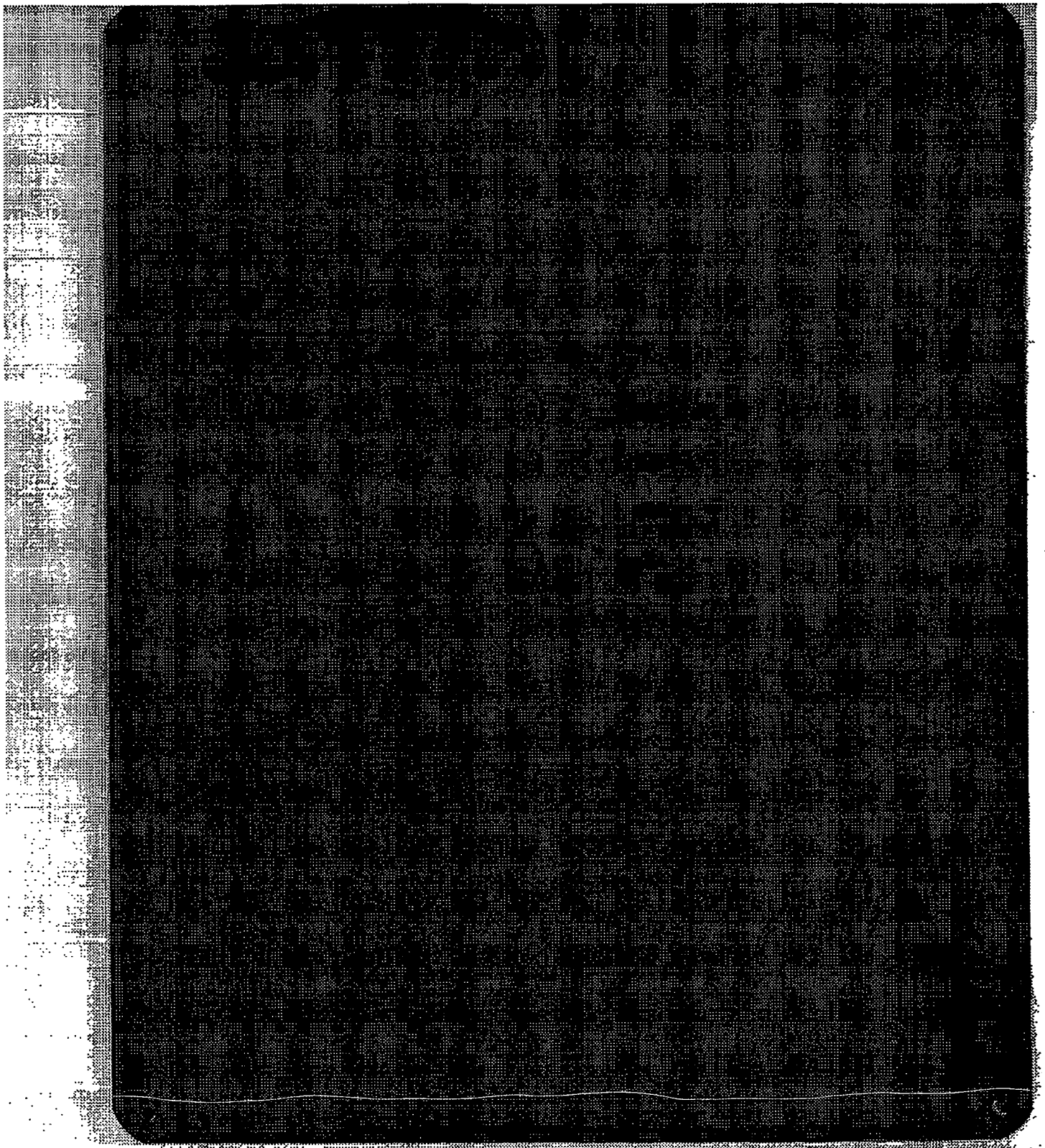
NAME: Todd Seeley

NOTES: Tot assay Publication
Quality result.!

Witnessed & Understood by me,

David Hall

Date	Initiated by <i>T Seeley</i>	Date
[REDACTED]	Recorded by <i>1</i>	[REDACTED]



|||

TITLE

Int.

Project No.

Book No.

71

From Page No.

18 flag-BUB3 pulls down BUB1/MAD2, try
Serial PCR deletion mapping to define
interacting regions. makes no sense to
clone if can be avoided. Kit says
eg. pole, A helps. Total efficiency.

p77 met

TTTTT

PA(n)

templ.

→ Could use this to myc or HA tag other
frags; this probably might keep frags
relatively small to avoid heterogeneity
in PCR prod. form.

Int Results: Great! Bands!!

Calibrated Molecular Weights of Prestained
SDS-PAGE Standards*

PROTEIN	HIGH RANGE	LOW RANGE	BROAD RANGE
Control #	#71605	#74177	#72807A
Myosin	205,000	—	208,000
β-galactosidase	118,000	—	115,000
Phosphorylase B	—	107,000	—
BSA	85,000	76,000	79,500
Ovalbumin	47,000	52,000	49,500
Carbonic anhydrase	—	36,800	34,800
Soybean trypsin inhibitor	—	27,200	28,300
Lysozyme	—	19,000	20,400
Aprotinin	—	—	7,200

* Molecular weights are of representative lots; actual weights may vary. The
lot-specific molecular weights are included with every vial.

Myosin
β-galactosidase
Bovine serum albumin
Ovalbumin
Carbonic anhydrase
Soybean trypsin inhibitor
Lysozyme
Aprotinin



Calc. sizes based on
rel. mobility.

MAD2
BUB3
FLAG BUB3
BUB1

27K
42K
46K
110K

Page No.

Witnessed & Understood by me,

David Duhl

Date

Invented by

T. Indley

Date

Recorded by

SEELEY
DECLARATION
EXHIBIT NO. 20

From Page No. _____

_____ prepared PCR methods
 chapters; prepared data to meet
 Rusty Williams; also patent

Recent Progress.

① huBUB1 kinase binds huBUB3. (Figure).

② BUB3 maps to a tumor-suppressor locus, 10q24

known genes @ 10q24

10148:148
10148:104(known
oncogenes)

- HOX 11 (haneobox).
- CMT 1 (NFK-B)

Wnt8B

FGF8

Fas

urokinase plasminogen activator (PLAU)

Suspected
tumor
suppressor

- MXI 1 — no mutations in 40 pancreatic
adenocarcinomas
- some mutations in prostate

Peter Austin
 Glioma

10q24 heavily studied by LOH in

- malignant meningioma
- prostate cancer
- glioma

in glioma; LOH mapped to D10S587-D10S216.
 D10S216 is flanking marker for linked
 huBUB3 STS!

③ Cell lines - some progress, some disappointing

④ huBUB3 antibody by direct DNA immunization.

vs animals.

Witnessed & Understood by me,

David Dahl

Date

Invented by

T. Imley

Date

Recorded by

n

SEELEY
DECLARATION
EXHIBIT NO. 21

S. cerevisiae Genes Required for Cell Cycle Arrest in Response to Loss of Microtubule Function

**M. Andrew Hoyt, Laura Totis,
and B. Tibor Roberts**
Department of Biology
The Johns Hopkins University
Baltimore, Maryland 21218

Summary

We have identified mutant strains of *S. cerevisiae* that fail to properly arrest their cell cycles at mitosis in response to the loss of microtubule function. New bud emergence and DNA replication (but not cytokinesis) occur with high efficiency in the mutants under conditions that inhibit these events in wild-type cells. The inability to halt cell cycle progression is specific for impaired microtubule function; the mutants respond normally to other cell cycle-blocking treatments. Under microtubule-disrupting conditions, the mutants neither achieve nor maintain the high level of histone H1 kinase activity characteristic of wild-type cells. Our studies have defined three genes required for normal cell cycle arrest. These findings are consistent with the existence of a surveillance system that halts the cell cycle in response to microtubule perturbation.

Introduction

The cell cycles of many mitotically growing eukaryotic cells can be considered to consist of a series of dependent events; that is, the initiation of many cycle-specific events is dependent on the successful execution of some previous event (reviewed in Hartwell and Weinert, 1989). For example, treatments that block DNA replication cause arrest in the S phase of the cycle (Slater, 1973; Osmani et al., 1988). Subsequent events such as mitosis, cytokinesis, and events associated with the start of the next cell cycle are inhibited. Likewise, disruption of microtubule structure usually causes cells to arrest at mitosis (Dustin, 1984). Numerous other dependency relationships have been described, especially in the yeasts where many cyclically occurring events can be inhibited by specific mutations (Pringle and Hartwell, 1981). It should be noted that in some rapidly dividing cell types, such as embryonic cells, some of these dependency requirements do not seem to operate (Hara et al., 1980; Raff and Glover, 1988).

Two models have been proposed to explain dependency relationships in the cell cycle (for discussion see Hartwell and Weinert, 1989). The first proposes that a cyclically occurring event is initiated when a preceding event has created a suitable product to be used as a substrate. For example, mitosis events may be initiated when a properly replicated and packaged set of chromosomes becomes available. The second model proposes the existence of specific feedback regulatory systems that monitor the execution of certain cyclical events. Should an event not be executed successfully, these systems act to inhibit the

initiation of subsequent events. Such surveillance-feedback mechanisms are said to constitute cell cycle "check-points" (Hartwell and Weinert, 1989).

The existence of a checkpoint mechanism that monitors the state of genomic DNA in *Saccharomyces cerevisiae* is strongly supported by the phenotypes of *rad9* mutant strains. When exposed to DNA-damaging treatments, eukaryotic cells pause in the G2 phase, presumably to allow repair of chromosomal damage prior to mitotic segregation. Weinert and Hartwell (1988) demonstrated that yeast *rad9* mutants have lost this capacity to pause in response to DNA damage. The sensitivity of *rad9* strains to DNA-damaging agents does not reflect a defect in the enzymatic capacity to repair lesions, but is probably due to premature mitotic segregation of damaged chromatids. The *rad9* alleles that cause this phenotype are of the loss-of-function variety (Weinert and Hartwell, 1990). Therefore, the precocious initiation of mitosis exhibited by a *rad9* cell can be understood as the consequence of loss of a surveillance-feedback system.

Treating mitotically growing eukaryotic cells with agents that disrupt microtubule structure causes arrest or pausing in the mitosis phase of their cell cycles (Dustin, 1984). In the yeast *S. cerevisiae*, disruption of microtubule structure can be accomplished with either addition of a benzimidazole compound to the growth medium (i.e., benomyl or nocodazole; Jacobs et al., 1988) or by a mutation that affects the stability of these structures (i.e., a tubulin mutation; Huffaker et al., 1988). Cells arrest at the large-budded stage containing a single nucleus with replicated but non-segregated chromosomes. Cytokinesis and events associated with the G1 phase of the next cell cycle (i.e., new bud emergence, nuclear DNA replication, and spindle pole body duplication) are inhibited.

In this paper we consider the possibility that mitotic arrest in response to loss of microtubule function is caused by an inhibitory feedback system, similar in principle to the *RAD9* system. We have identified mutant strains of *S. cerevisiae* that fail to arrest their cell cycles in response to the loss of microtubule function. New bud emergence and DNA replication (but not cytokinesis) occur with high efficiency in these mutants under conditions that arrest wild-type cells. The mutants respond normally to other cell cycle-blocking treatments.

In eukaryotic cells, mitotic cycle progression is controlled by the cyclical activation of the $p34^{cdc2/CDC28}$ kinase (Murray and Kirschner, 1989; Nurse, 1990). This activity is required to drive cells into mitotic metaphase. Wild-type yeast cells arrested at mitosis with nocodazole have been observed to exhibit elevated levels of histone H1 kinase activity, a measure of $p34^{cdc2/CDC28}$ function (Langan et al., 1989). In extracts from *Xenopus* eggs, progression from mitotic metaphase to anaphase was shown to require the destruction of $p34^{cdc2/CDC28}$ activity (Murray et al., 1989). A system that inhibits cell cycle progression at mitosis might possibly function by regulating the activity of $p34^{cdc2/CDC28}$. To explore this possibility, we also analyzed histone H1

Serial No. 10/084,700
Filed 02/27/2002
SEELEY DECLARATION
EXHIBIT NO. 21

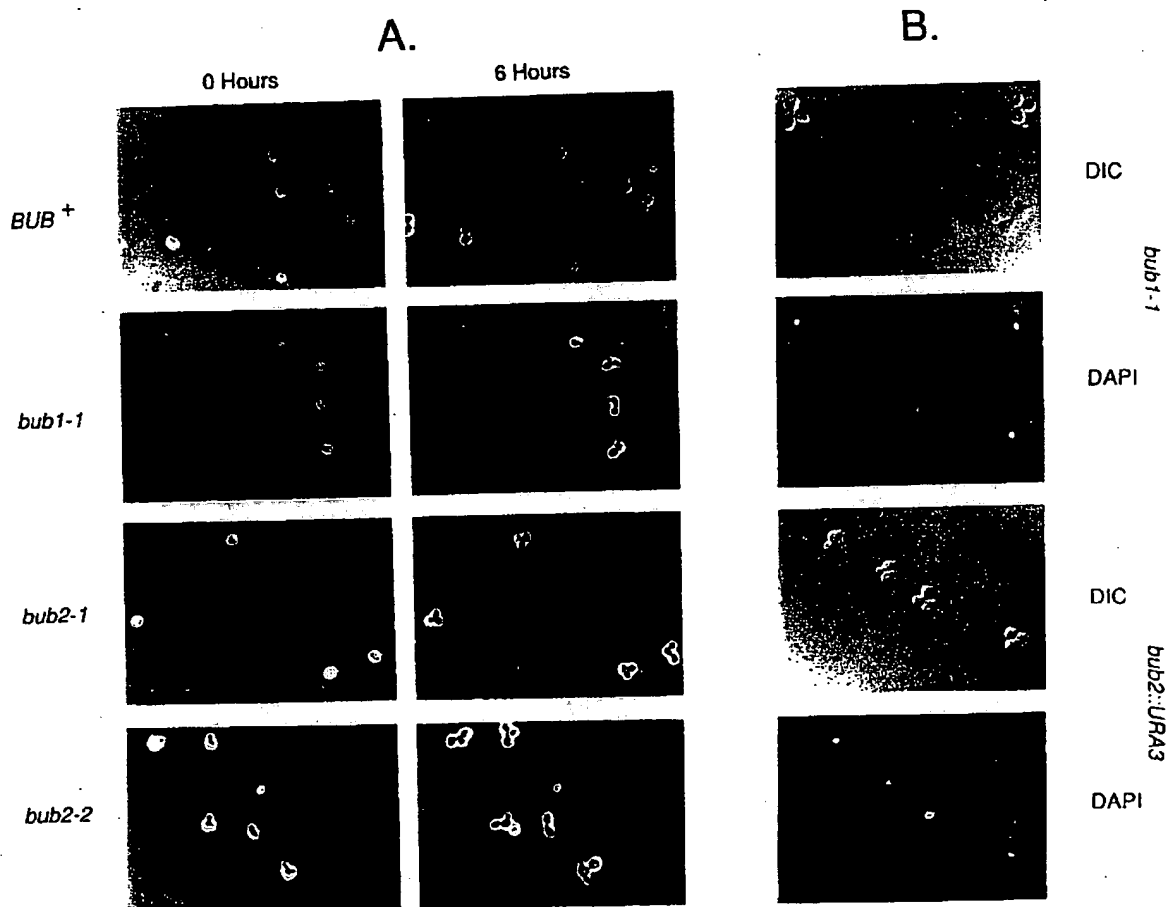


Figure 1. Microscopic Examination of Cells Grown in Benzimidazole

(A) Time-lapse photography of cells on solid medium containing benomyl: α factor-synchronized cells were released onto YPD agar slabs containing 70 μ g/ml benomyl. The fields were photographed immediately (0 hr; left vertical column) and after 6 hr at 26°C (right vertical column). (B) *bub* cells grown in liquid medium containing nocodazole: α factor-synchronized cells were released into liquid YPD containing 15 μ g/ml nocodazole. After 6 hr at 26°C cells were sonicated to break up clumps and fixed. They were then stained with the DNA-specific fluorescent dye 4,6-diamidino-2-phenylindole and analyzed microscopically with differential interference contrast optics (DIC) and epifluorescent illumination (DAPI). Note the presence of only a single nucleus, as evidenced by a brightly stained mass of DNA, in each multibudded cell. Yeast strains: *BUB*⁺ (MAY589), *bub1-1* (MAY1726), *bub2-1* (MAY1675), *bub2-2* (MAY1568), *bub2::URA3* (MAY2055).

kinase activity in our mutant cells and found its regulation to be defective.

Results

Mutants That Recover Poorly from Loss of Microtubule Structure

We anticipated that hypothetical mitosis checkpoint mutants may be particularly deficient in their ability to recover from a complete but transient block to microtubule assembly. If unable to properly pause the cell cycle in response to loss of microtubule function, the execution of events out of sequence may result in lethality. Colonies from mutagenized cells were replica plated to medium containing enough benomyl to cause a total loss of all microtubule structure (70 μ g/ml). (Note that for technical reasons we use different, but related benzimidazole compounds in dif-

ferent experimental growth media; nocodazole was used in liquid medium and benomyl in agar-based medium.) After 20 hr on benomyl, cells were replica transferred back to rich medium. Potential mutants were those that recovered poorly from this regimen relative to recovery from 20 hr on an agar-only substrate. *S. cerevisiae* cells starved for nutrients arrest in late G1 just prior to the "START" event of a new cell cycle (Pringle and Hartwell, 1981). The agar-only control therefore added some specificity for mutants sensitive to arrest only at mitosis, a point that we address more rigorously below.

Among the mutants that recover poorly from benomyl treatment, those with altered cell cycle regulation were identified by microscopic observation. Cells were synchronized in the G1 phase by treatment with mating pheromone (α factor). At least 90% of the cells in each tested culture assumed an unbudded morphology. The cells

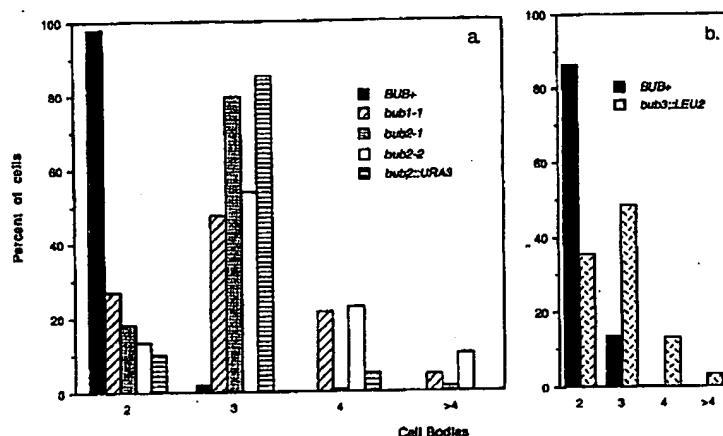


Figure 2. Quantitation of Arrest Morphology on Solid Medium Containing Benomyl

(a) α factor-synchronized cells were released onto YPD agar slabs containing 70 μ g/ml benomyl. After 6 hr at 26°C, the slabs were examined microscopically. The percentage of groupings containing the indicated numbers of cell bodies was determined. A single large-budded cell has a value of two cell bodies.

(b) Same as (a), except after incubation for 20 hr at 24°C.

Yeast strains are the same as for Figure 1 plus *bub3::LEU2* (MAY2072).

were then released from G1 arrest onto solid medium at 26°C containing 70 μ g/ml benomyl. Wild-type cells progressed through the cell cycle until mitosis, at which point they arrested as large-budded cells (Figure 1). Seven out of 194 mutants that recovered poorly from benomyl arrest did not stop as singly budded cells. Instead, the mutant cells usually produced at least one more bud within the 6 hr period analyzed (Figure 1). The results of this type of assay are summarized quantitatively in Figure 2. It should be noted that the aberrant extra budding by the mutant cells occurs at a slower rate than the typical budding schedule of an untreated cell. The wild-type doubling time at 26°C (in the absence of benomyl) is approximately 2 hr. We have named these mutants *bub* (for "budding uninhibited by benzimidazole"), and three (*bub1-1*, *bub2-1*, and *bub2-2*) were chosen for further study.

The three *bub* mutations also cause sensitivity to what are normally sublethal concentrations of benomyl in the medium. This phenotype is somewhat subtle but useful for genetic analyses. They are typically sensitive to benomyl at 10 μ g/ml, while wild-type haploids are resistant to approximately 15 μ g/ml. Contrast this to two mutations that destabilize yeast microtubule structures: *tub1-1*, a mutant allele of the major α -tubulin gene, and *cin1* (Stearns and Botstein, 1988; Hoyt et al., 1990; Stearns et al., 1990). Although *tub1-1* and *cin1*-null mutant strains are extremely sensitive to benomyl, being unable to form colonies on plates containing 1 μ g/ml, they recovered efficiently after a 20 hr treatment with 70 μ g/ml (unpublished data). All pairwise diploid combinations were constructed using *bub* and *BUB+* haploids and assayed for benomyl resistance. All three mutants were recessive for both the hypersensitivity-to-low-benomyl phenotype and the continued budding-in-high-benomyl phenotype. The *bub2-1* and *bub2-2* mutants failed to complement for benomyl resistance, and subsequent linkage analysis revealed them to be allelic (see below). *bub1-1* is a mutant allele of a distinct gene.

Continued budding in the presence of benomyl might be an artifactual response to this agent. To rule out this possibility, we sought to perturb microtubule function mutationally. Double mutants were constructed between

bub1 or *bub2* alleles and cold-sensitive β -tubulin (*tub2*) alleles (Huffaker et al., 1988). The single and double mutants were arrested in G1 with α factor, then released onto medium prechilled to 11°C. The *tub2* single mutants arrested growth primarily as large-budded cells (Table 1). The double mutants aberrantly continued bud production at 11°C. Mutant alleles of *cin1* resemble tubulin mutations in that they cause yeast microtubule structures to become cold sensitive (Hoyt et al., 1990). A *cin1-bub2* double mutant continued budding at 11°C, while the *cin1* single mutant did not (Table 1). Therefore, the failure of *bub* mutants to arrest cell cycle progression occurs independently of the method used to disrupt microtubule function.

Benzimidazole-Induced Lethality of *bub* Mutants Requires Cell Cycle Progression

If our mutant strains are indeed defective in cell cycle arrest and not microtubule function, then benzimidazole-induced lethality should be a function of continued cell cycle progression, not microtubule disruption. To test this prediction, wild-type and mutant cultures were arrested in G1 with α factor and released into liquid medium containing nocodazole alone or nocodazole plus either α factor or the DNA synthesis inhibitor hydroxyurea. The pres-

Table 1. Aberrant Cell Cycle Arrest of *tub2* and *cin1* Caused by *bub*

Strain	Genotype	Cell Bodies	
		2	≥ 3
MAY1067	<i>tub2-104</i>	95%	5%
MAY1834	<i>tub2-104 bub2-1</i>	21%	79%
MAY1760	<i>tub2-403</i>	78%	22%
MAY1858	<i>tub2-403 bub1-1</i>	43%	57%
MAY1843	<i>tub2-403 bub2-1</i>	45%	55%
MAY933	<i>cin1::HIS3</i>	85%	15%
MAY2128	<i>cin1::HIS3 bub2::URA3</i>	25%	75%

α factor-synchronized cells were released onto agar-based medium at 11°C. After 24 hr the percentage of groupings with the indicated number of cell bodies was determined.

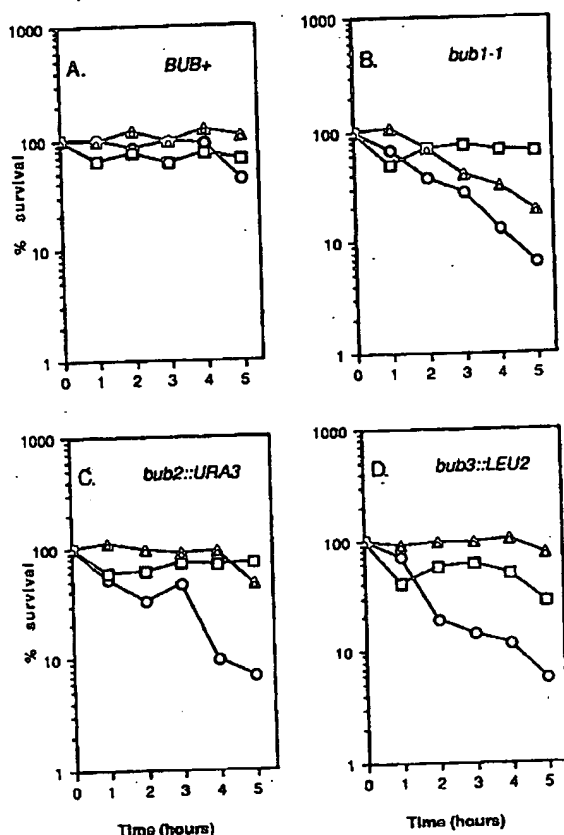


Figure 3. Survival of Cells in Nocodazole-Containing Media
 α factor-synchronized cells of the indicated genotype were released into YPD at 26°C containing nocodazole only (circles) or nocodazole plus either α factor (squares) or hydroxyurea (triangles). Aliquots were removed at the indicated time points and titered for viable colony-forming units on YPD agar. Yeast strains are the same as for Figures 1 and 2.

ence of α factor or hydroxyurea prevents cells in G1 from progressing to mitosis. Aliquots were removed at intervals and titered for survivors on permissive medium (Figure 3). Although its growth was inhibited by these treatments, the wild-type culture did not lose viability in nocodazole. Following a lag period, the *bub* mutants displayed a loss of viability in the nocodazole-only culture. The addition of either α factor or hydroxyurea, however, prevented the nocodazole-induced lethality, except for *bub1-1* where hydroxyurea only partially (but reproducibly) rescued. This finding indicates that loss of microtubule structure is not sufficient to kill the *bub* strains; active cell cycle progression beyond S phase is additionally required. Presumably, the cells die when they pass a mitotic checkpoint, a process that was prevented in this experiment by either α factor or hydroxyurea.

bub Mutants Continue DNA Replication Despite Loss of Microtubules

When *S. cerevisiae* cells pass the point in late G1 defined as START, they produce a daughter bud, initiate DNA replication, and duplicate their spindle poles within a short

time period (Pringle and Hartwell, 1981). The continued budding in benomyl indicated that the *bub* mutants may be aberrantly traversing START. As another criterion for this event, we compared DNA replication in our strains. Log-phase and nocodazole-treated cells were fixed, stained for DNA with propidium iodide, and examined by flow cytometry (Figure 4). For both wild-type and *bub* mutants, the log-phase cultures contained cells with either a G1 (pre-S phase) or G2 (post-S phase) DNA content. Nocodazole treatment for 4 hr shifted most of the wild-type cells into the G2 DNA peak (DNA was replicated once but not segregated). Nocodazole treatment of both the *bub1* and *bub2* mutant cells resulted in the appearance of cells with a higher than G2 DNA content. This indicated that additional DNA replication was not inhibited by nocodazole in the *bub* strains as it was in wild type.

The *bub* Phenotype Is Specific for Impaired Microtubule Function

The above results suggest that *bub1* and *bub2* mutants traverse START despite a complete block to the previous mitosis. This phenotype could be due to a defect in START regulation, as opposed to a defect in responding to impaired microtubule function. Specificity for microtubule function is suggested by the following observations. Other treatments that block the cell cycle of wild-type cells also block *bub1* and *bub2* strains. Treatment with the mating pheromone α factor caused a normal G1 arrest (greater than 90% unbudded shmoo-shaped cells). This contrasts with the *far1* and *fus3* mutants that fail to arrest their cycles in response to α factor (Chang and Herskowitz, 1990; Elion et al., 1990). The DNA synthesis inhibitor hydroxyurea also elicited a normal S phase cell arrest in the *bub* strains (data not shown). Like wild type, *bub* mutant cells arrested with two cell bodies and did not continue budding in hydroxyurea. In fact, both hydroxyurea and α factor prevent *bub* lethality caused by nocodazole, presumably by preventing progression of the cell cycle through mitosis (see above). *rad9* mutants fail to pause their cell cycles in response to DNA damage and are therefore hypersensitive to gamma irradiation (Weinert and Hartwell, 1988). Neither *bub1* nor *bub2* was any more or less sensitive than wild type to gamma irradiation (data not shown).

Mutant alleles of three of the *S. cerevisiae* cell division cycle genes (*cdc16*, *cdc20*, and *cdc23*) cause a similar terminal arrest phenotype as benzimidazole-induced microtubule disruption (Byers and Goetsch, 1974; Pringle and Hartwell, 1981). Cells arrest with a large-budded mononucleate morphology containing replicated but nonsegregated chromosomes. Microtubule structures, however, are not disassembled in these arrests. *cdc14* and *cdc15* mutations cause arrest at a subsequent step (or steps) in the nuclear division pathway. We investigated whether the arrest phenotype of these *cdc* mutants is dependent on the function of *BUB1* and *BUB2* as is the arrest phenotype of benzimidazole. Temperature-sensitive alleles of all five of these *CDC* genes were used to construct all combinations of double mutants with *bub1* and *bub2* alleles. Liquid cultures of the double mutants and corresponding *cdc* single mutants were grown at the permissive temperature of 23°C and shifted to 37°C for 3.5 hr. After fixation, the

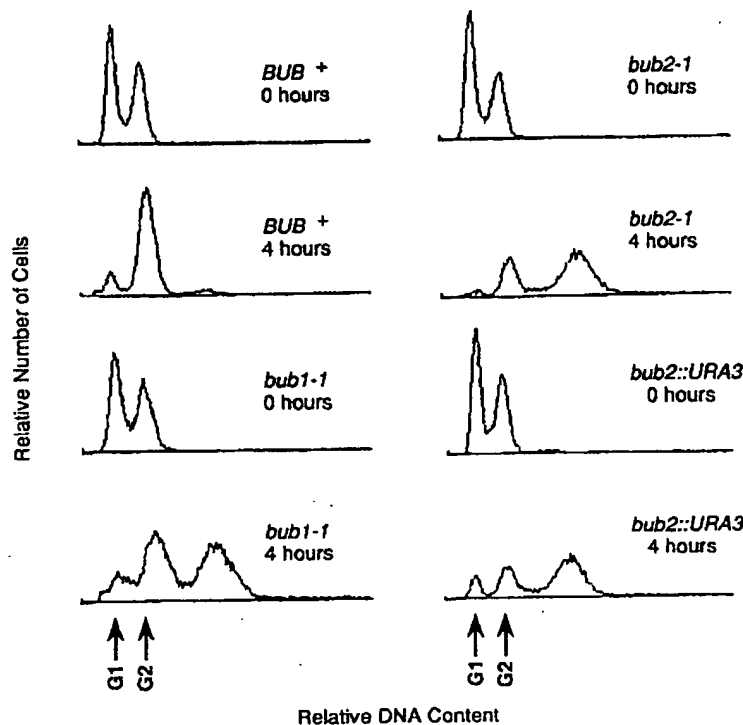


Figure 4. Flow Cytometric Analysis of DNA Content

Log-phase cells of the indicated genotypes were treated with nocodazole for 4 hr at 26°C. Samples were taken both before (0 hr) and after treatment (4 hr), fixed, stained for DNA, and analyzed by flow cytometry. The arrows labeled G1 and G2 indicate the positions of cells containing pre-S phase and post-S phase DNA contents, respectively.

cells were examined for morphology and DNA content by flow cytometry. Neither the double mutants nor any of the *cdc* single mutants showed any evidence of continued budding or DNA overreplication at 37°C.

Cytokinesis Remains Dependent on Microtubule Function in *bub* Mutants

When G1 arrested *bub* mutants were released into nocodazole-containing liquid medium, multibudded mononucleate cells appeared at a high frequency (Figure 1B). These clusters remained intact after vigorous sonication, indicating that cell separation had not occurred. In *S. cerevisiae*, cell separation is temporally distinct from and functionally dependent on cytokinesis (Hartwell, 1971). Cells that have undergone cytokinesis but not separation can be recognized by their sensitivity to the cell wall-degrading enzyme glusulase. Budded *bub* cells generated by nocodazole treatment were digested with glusulase and compared with undigested samples. The percentage of budded cells in the samples decreased only slightly after glusulase treatment (down 12% and 20% for *bub1-1* and *bub2::URA3*, respectively), indicating that most of the cells had not undergone cytokinesis. Therefore, in *bub* mutants, execution of cytokinesis remains dependent on some aspect of microtubule function, while execution of START no longer shows this dependency.

bub Mutants Have Altered Histone H1 Kinase Activity

In vitro histone H1 kinase activity reflects the intracellular activity of p34^{cdc2/cdc28}, the major effector of cell cycle progression (Langan et al., 1989; Wittenberg and Reed,

1988). We considered the possibility that the *BUB* gene products may affect the cell cycle by regulating this activity. Cells were synchronized in G1 with α factor and released into liquid medium. At intervals, samples were removed and crude cytosolic extracts were prepared and assayed for histone H1 kinase levels (Figure 5). As has been reported by others, the G1 arrested samples displayed relatively low kinase activity (Wittenberg and Reed, 1988). When wild-type cells were released into medium without nocodazole, the activity rose from this minimal value, peaking at 1.25 hr. Between 1.25 and 1.5 hr, a sharp decrease was observed, corresponding to the approximate time of mitosis (data not shown). The pattern then repeated itself, although the rise in the second cycle was not as high, presumably due to loss of synchrony. In the absence of nocodazole, the *bub1* and *bub2* strains showed histone H1 kinase cycling behavior that was similar to wild type (data not shown). Wild-type cells released from G1 arrest into nocodazole-containing medium initially followed the same pattern, but the characteristic M phase drop in activity did not occur. Instead, activity continued to rise and remained at a high level through the final 3 hr time point. Release of the *bub1* and *bub2* strains from G1 into nocodazole resulted in a rise of kinase activity, peaking at 1.25–1.5 hr, followed by a gradual decrease. The sharp decline characteristic of mitosis was not observed, but the *bub* mutants clearly could neither achieve nor maintain the high levels seen in nocodazole-arrested wild-type cells.

Cloning, Sequencing, and Disruption of *BUB2*

We isolated a clone of the wild-type *BUB2* gene by complementation of the mutant phenotypes. A library of *S. cere-*

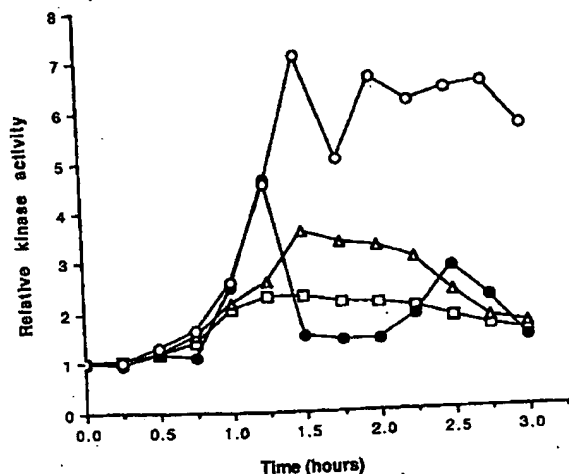


Figure 5. Histone H1 Kinase Activity Assay
a factor-synchronized cells were released into YPD, and aliquots were removed at 15 min intervals. Crude cytosolic extracts were prepared and assayed for the ability to phosphorylate bovine histone H1 as described in Experimental Procedures. Measured activity for each strain was normalized to the level observed at 0 min after release from a factor arrest. Symbols: closed circles, *BUB*⁺, no nocodazole; open circles, *BUB*⁺ plus nocodazole; squares, *bub1-1* plus nocodazole; triangles, *bub2::URA3* plus nocodazole.

visiae DNA in a *CEN* vector was transformed into a *bub2-1* strain, and transformants resistant to 10 µg/ml benomyl were identified. Cells from one transformant had also lost the budding-uninhibited-by-benzimidazole phenotype. We demonstrated that the suppressing plasmid con-

tained DNA from the bona fide *BUB2* locus by using a fragment derived from it to direct chromosomal integration of a detectable marker gene (see Experimental Procedures). Subsequent tetrad analysis revealed that the integrated marker was linked to both *bub2-1* and *bub2-2*. The cloned DNA also suppressed the mutant phenotypes of *bub2-2*.

Subcloning was used to narrow the location of the gene providing *BUB2* complementing activity (Figure 6A). DNA sequencing revealed a single open reading frame in this region (Figure 7). A polypeptide product of 306 amino acids is predicted. Using the FASTA homology search program (Pearson and Lipman, 1988), no significant similarities to other polypeptide sequences in either the GenPept or SWISS-PROT data bases were found (release numbers 64.3 and 17, respectively). A sequence in the region 5' to *BUB2* proved to be the 3' end of the *PET9* gene (Adrian et al., 1986). This finding localizes the map position of *BUB2* to the left arm of chromosome II.

A null allele of *bub2* was constructed by deleting 202 bp of coding sequence and replacing it with DNA encoding *URA3*. Haploids carrying the *bub2::URA3* allele (see Experimental Procedures) were viable and grew at wild-type rates, indicating that *BUB2* is nonessential for viability. *bub2::URA3* strains behaved identically to the original two mutants. When the *bub2::URA3* strains were crossed, the mutant phenotypes cosegregated with the *URA3* marker.

Identification of *BUB3*, a Suppressor of *bub1-1*

The same cloning-by-complementation strategy was used in an attempt to isolate the *BUB1* gene. Seven plasmid clones, containing overlapping but unique DNA inserts,

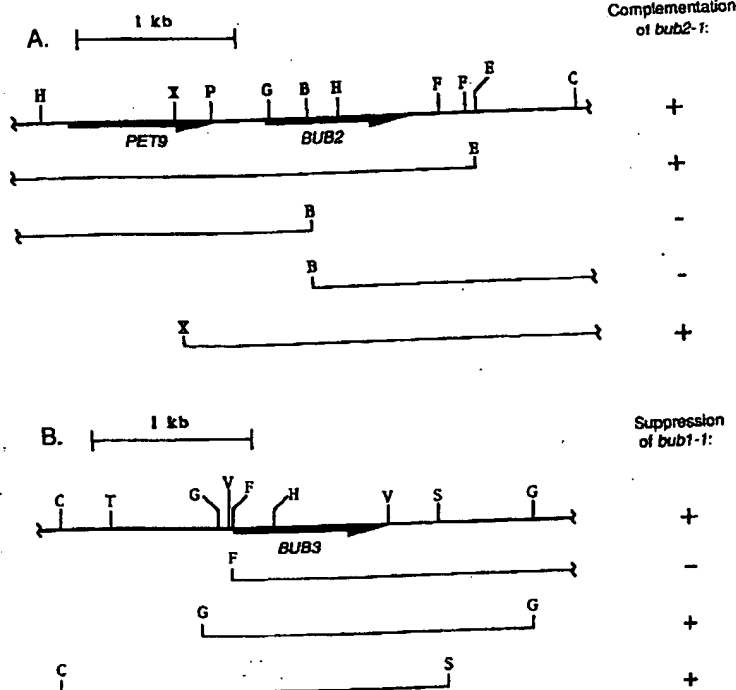


Figure 6. Restriction Enzyme Site Maps of the *BUB2* and *BUB3* Loci

(A) The *BUB2* locus. (B) The *BUB3* locus. Thickened regions of the map indicate the extent of the indicated open reading frame with the arrowhead directed toward the 3' end. Lines beneath the restriction maps indicate DNA that is contained within the subclone tested. A plus or a minus sign indicates whether the subclone suppressed the *bub* phenotypes of (A) *bub2-1* or (B) *bub1-1*. Restriction enzyme sites: B, BamHI; C, ClaI; E, EcoRI; F, SphI; G, BglII; H, HindIII; P, PstI; S, SstI; T, StuI; V, EcoRV; X, XbaI.

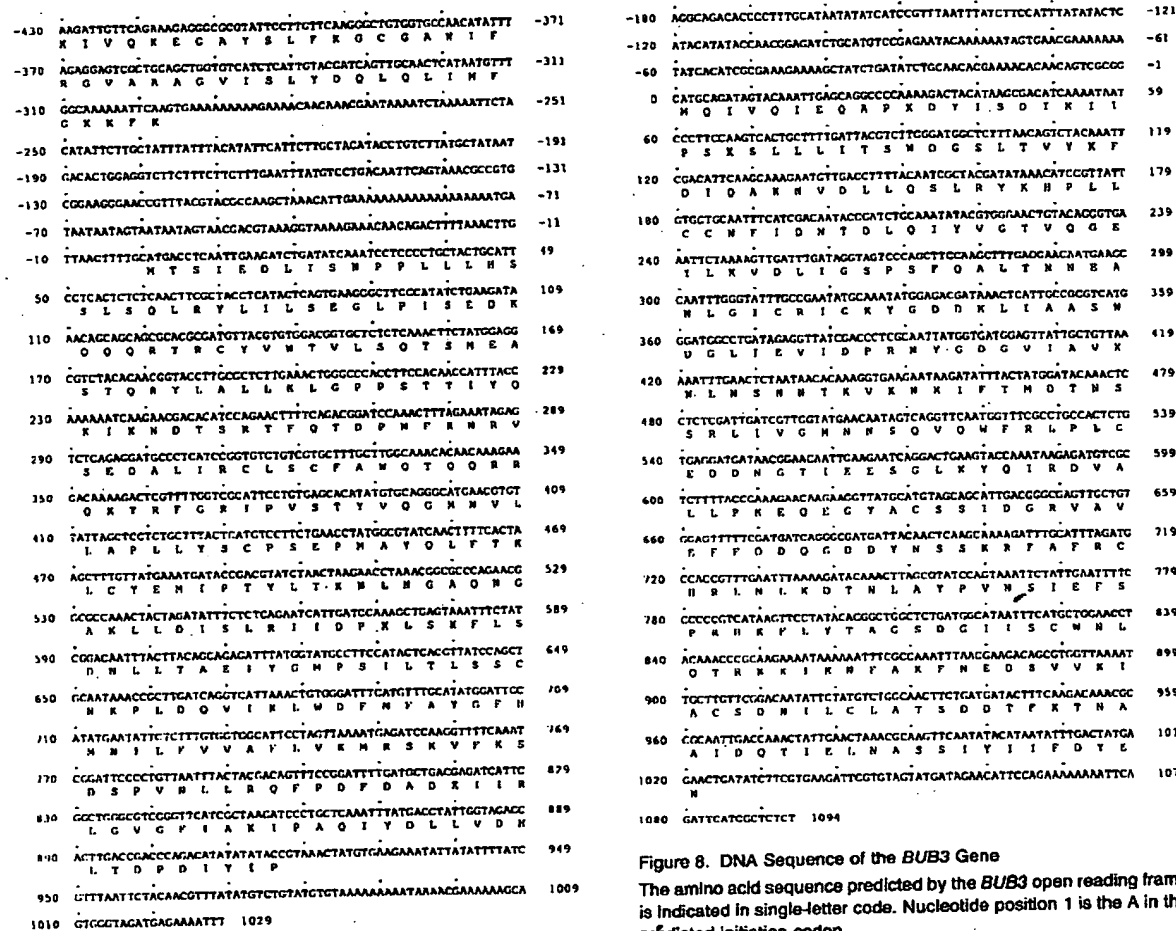


Figure 7. DNA Sequence of the *BUB2* Gene

The amino acid sequence predicted by the *BUB2* open reading frame is indicated in single-letter code. Nucleotide position 1 is the A in the predicted initiation codon. Nucleotides -430 through -314 represent the 3' end of the *PET9* open reading frame.

were isolated from two *CEN* libraries. All seven suppressed both the benomyl sensitivity and the *bub* phenotypes of *bub1-1*. However, two distinct integration linkage experiments (see Experimental Procedures) revealed that this cloned chromosomal locus was unlinked from the site of the *bub1-1* locus. Therefore, this new locus represents an extra copy suppressor of *bub1-1*. We have named the gene at this locus *BUB3* because mutations result in *bub*-like phenotypes (see below).

Subcloning was used to identify the size of the smallest DNA region carrying the *BUB3* gene (Figure 8B). This region was found to contain a single open reading frame that encodes a predicted polypeptide product of 341 amino acids (Figure 8). No significant similarities to either *BUB2* or other polypeptide sequences in the GenPept or SWISS-PROT databases were observed. A radiolabeled *BUB3* probe hybridized to chromosome XV on a Southern blot of pulse-field gel-separated chromosomes (data not shown).

Figure 8. DNA Sequence of the *BUB3* Gene

The amino acid sequence predicted by the *BUB3* open reading frame is indicated in single-letter code. Nucleotide position 1 is the A in the predicted initiation codon.

Tight linkage (49 parental ditype: 0 nonparental ditype: 6 tetratype or 5.5 cM) was observed to the centromere-proximal locus *PHO80* (Lange and Hansche, 1980). High and low stringency Southern blots to yeast genomic DNA suggest that the *BUB3* sequence is unique in the genome (data not shown).

A null allele of *bub3* was constructed by deleting 1129 bp of its open reading frame and replacing it with DNA encoding *LEU2* (see Experimental Procedures). *bub3*-null haploid spores were generated from a sporulated *bub3::LEU2/BUB3*⁺ diploid. The *Leu*⁺ spores grew extremely slowly relative to the *Leu*⁻ spores. This slow growth phenotype reverted rapidly. The small spore clone colonies contained both slow and fast growing cells; after passaging, the fast growers predominated. Observations of *bub3* phenotypes are therefore subject to the qualification that suppressing mutations apparently exist in all cultures. *bub3* cultures were also observed to contain large numbers of dead cells. Nonetheless, *bub3::LEU2* cultures display the mutant phenotypes characteristic of *bub1* and *bub2*. They are hypersensitive to benzimidazoles (at 7.5 µg/ml benomyl), and continue budding (Figure 2) and die rapidly (Figure 3) in high concentrations of these agents. Like *bub1*

and *bub2*, death in nocodazole was prevented by either a factor or hydroxyurea.

Discussion

A Microtubule Surveillance-Feedback System

We have identified three *S. cerevisiae* genes whose products are required for cell cycle arrest in response to loss of microtubule function. The failure to arrest is specific for microtubule disruption; *bub* mutants respond normally to mating pheromone and DNA synthesis inhibition as well as to mutational blocks to the cell cycle. When mitosis is blocked in wild-type cells, events associated with START of the next cell cycle (bud emergence and DNA replication) are inhibited. In *bub* mutants, these events are not restrained. In the simplest formulation, the *bub* cells are continuing cell cycle progression under conditions in which mitosis is blocked. Alternatively, when unable to proceed through mitosis, *bub* cells may be skipping to another point in the cell cycle, a point from which START events can be initiated. Mutant forms of $p34^{cdc2/CDC28}$ in *Schizosaccharomyces pombe* that can cause this type of defect have been described (Broek et al., 1991). These mutants arrest in G2, but when transferred back to permissive conditions resume growth at START. Whichever of these two possibilities is correct, however, it is clear that *bub* cells cannot maintain the M phase arrested state. Mutants with similar phenotypes (*mad*) have been identified by another group and are reported in the accompanying paper (Li and Murray, 1991). Comparison of the cloned genes reveals that *MAD2* is distinct from *BUB2* and *BUB3*. Genetic tests have shown that *MAD2* is not equivalent *BUB1* (P. Meluh, personal communication).

Our findings are consistent with the existence of a specific cell cycle feedback mechanism that acts when microtubule function is compromised. Presumably, the *bub* strains are actually defective for some aspect of this system. A priori, a surveillance-feedback system must possess distinct sensing and signaling functions. At present we are unable to distinguish one or both of these possible functions for the three identified *BUB* genes. The *BUB* genes join a group of yeast genes recently recognized for their requirement for normal cell cycle arrest. *FAR1* and *FUS3* are required for cell cycle arrest by mating pheromone and *RAD9* for arrest by DNA damage (Chang and Herskowitz, 1990; Elion et al., 1990; Weinert and Hartwell, 1988). The specificity for the arresting treatment exhibited by this family suggests the existence of numerous checkpoint surveillance systems. At some level, however, these systems must feed information into a common cell cycle regulatory pathway (see below).

It is reasonable to presume that the hypothetical "BUB" surveillance system (or systems) does not exist simply for the purpose of responding to complete loss of microtubule function. In a normally cycling cell, BUB may function routinely to ensure the correct order of events. Since *bub2* and *bub3* are not essential for viability, their ordering functions are either not essential under normal growth conditions, or are provided by alternative systems in their absence. The function of BUB is specific for mitotic spindle

defects, although these defects need not be as extreme as total microtubule loss. In *S. cerevisiae*, centromere mutations can cause a cis-acting reduction in chromosome transmission fidelity. Some of these mutations cause a cell cycle delay at or near M phase (F. Spencer and P. Hieter, submitted). In cultured newt lung cells, single chromosomes are occasionally found to lag in their attachment to the mitotic spindle (Rieder and Alexander, 1989). In these cells, this lag in attachment is accompanied by a delay in cell cycle progression. BUB, or a similar system, could be responding to these types of less severe spindle malfunctions. It is worth noting that although our *bub1* and *bub2* strains grew well in the absence of benzimidazole, phenotypes consistent with aberrant mitoses or accelerated START have been observed (unpublished data). In genetic crosses with these strains, we often saw evidence of an extra chromosome or whole sets of chromosomes being contributed by the *bub* parent. Occasionally, DNA analysis by flow cytometry revealed that much of a *bub* culture had increased in ploidy during mitotic growth.

Cytokinesis remains dependent on microtubule function in the *bub* mutants. We propose that this reflects the non-sequential nature of the cell cycle pathway after mitosis. Although cytokinesis occurs after mitosis, it is not a prerequisite for START in yeast; mutations that block cytokinesis (*cdc3*, -10, -11, and -12) do not inhibit START (Hartwell, 1971; Hartwell et al., 1974). Therefore, cytokinesis may lie on a separate branch of the cell cycle, a branch that is still dependent on some aspect of microtubule function despite the absence of BUB.

BUB Regulation of the Cell Cycle

We have determined that histone H1 kinase activity is defectively regulated in *bub1* and *bub2* strains. Wild-type cells blocked at mitosis accumulate high levels of this activity (Langan et al., 1989). The *bub* strains fail to maintain high levels of kinase activity in the presence of nocodazole. The function of BUB gene products may be direct regulation of the kinase activity of $p34^{cdc2/CDC28}$. Alternatively, BUB might affect this kinase indirectly, or the observed reduction of kinase activity in the *bub* strains may reflect a secondary consequence of cell cycle progression.

The kinase activity of $p34^{cdc2/CDC28}$ appears to be subject to numerous regulatory controls (Murray and Kirschner, 1989; Nurse, 1990). For yeast (and perhaps all eukaryotes) its activity is essential at two different steps in the cell cycle: the transition from G1 to S phase (START) and for entry into mitosis (Reed, 1991). The $p34^{cdc2/CDC28}$ kinase is activated by its association with the "G1 cyclins" at START and by distinct "M phase cyclins" at mitosis. Degradation of M phase cyclins and hence $p34^{cdc2/CDC28}$ kinase activity is required for the transition from metaphase to anaphase; the addition of a degradation-resistant cyclin to *Xenopus* egg extracts inhibits the metaphase/anaphase transition. Therefore, a possible role for the BUB gene products is to prevent M phase cyclin degradation when microtubule function is compromised. This is in contrast to *FAR1* and *FUS3*, which may keep the cell from passing START by antagonizing the action of the G1 cyclins (Chang and Herskowitz, 1990; Elion et al., 1990). It is unclear how *RAD9*

Table 2. Yeast Strains

Strain Number	Relevant Genotype
MAY589	α <i>BUB</i> ⁺
MAY 591	α <i>BUB</i> ⁺
MAY933	α <i>cin1::HIS3</i>
MAY1087	α <i>tub2-104</i>
MAY1210	α/α <i>BUB</i> ⁺ <i>BUB</i> ⁺
MAY1569	α <i>bub2-2</i>
MAY1575	α <i>bub2-1</i>
MAY1726	α <i>bub1-1</i>
MAY1760	α <i>tub2-403</i>
MAY1786	α <i>cdc20-1 bub1-1</i>
MAY1790	α <i>cdc16-1 bub1-1</i>
MAY1834	α <i>tub2-104 bub2-1</i>
MAY1843	α <i>tub2-403 bub2-1</i>
MAY1858	α <i>tub2-403 bub1-1</i>
MAY1877	α <i>pho80::URA3</i> (original allele from L. W. Bergman)
MAY1908	α <i>cdc23-1 bub1-1</i>
MAY1966	α <i>BUB3:URA3:BUB3</i>
MAY1988	α <i>BUB2:URA3:BUB2</i>
MAY2055	α <i>bub2::LEU2</i>
MAY2072	α <i>bub3::LEU2</i>
MAY2085	α <i>cdc14</i>
MAY2086	α <i>cdc14 bub2::URA3</i>
MAY2089	α <i>cdc15-2</i>
MAY2094	α <i>cdc16-1 bub2::URA3</i>
MAY2097	α <i>cdc23-1 bub2::URA3</i>
MAY2098	α <i>cdc23-1</i>
MAY2099	α <i>cdc20-1 bub2::URA3</i>
MAY2100	α <i>cdc20-1</i>
MAY2101	α <i>cdc16-1</i>
MAY2106	α <i>cdc15-2 bub2::URA3</i>
MAY2110	α <i>cdc14 bub1-1</i>
MAY2112	α <i>cdc15-2 bub1-1</i>

might induce cell cycle arrest; perhaps it acts by inhibiting the accumulation of M phase cyclins or by otherwise down-regulating p34^{cdc2/cdc28} kinase activity. It will be interesting to test for possible interactions between the *BUB* genes and the M phase cyclins when the appropriate cyclin mutants become available.

Experimental Procedures

Yeast Strains and Media

The yeast strains used in these experiments are derivatives of S288C and are listed in Table 2. *bub* mutant alleles were backcrossed to wild type at least twice. Rich (YPD), minimal (SD), and sporulation media were as described (Sherman et al., 1983). Benomyl (Du Pont) was added to solid medium from a 10 mg/ml stock in dimethyl sulfoxide. For complete disruption of microtubule function, it was added to a final concentration of 70 μ g/ml. Minimal doses, to assess benomyl sensitivity, were in the range of 7.5 to 12.5 μ g/ml. Nocodazole (Sigma) was added to liquid YPD (pH 4.0) to 15 μ g/ml from a 3.3 mg/ml stock in dimethyl sulfoxide. This concentration caused a complete loss of microtubule-based structures as judged by immunofluorescence microscopy (data not shown). The mating pheromone α factor (Sigma) was used in YPD (pH 4.0) at 6–8 μ g/ml. The DNA synthesis inhibitor hydroxyurea (Sigma) was added to medium to 0.1 M.

Screen for *bub* Mutants

Strain MAY589 was mutagenized with ethyl methanesulfonate to 10% survival and plated for single colonies on YPD at 26°C. Developed colonies were replica transferred to YPD plates containing benomyl at 70 μ g/ml and to plates containing agar only. After 20 hr at 26°C, replicas were made of these two plates onto YPD at the same tempera-

ture. Mutant cells that formed healthy colonies coming from the agar-only plate but not the benomyl-containing plate were selected for further analysis.

To assess the ability to halt bud emergence in the presence of benomyl, cells were first synchronized in the G1 phase by one of two methods. For crude testing of many strains at once, cells were grown to saturation in liquid cultures for roughly 48 hr (starvation arrests in G1). For rigorous assays, log-phase cells in YPD were treated with α factor for 4 hr at 26°C. After α factor treatment, cells were examined to ensure that at least 80% were unbudded. Following arrest in G1, cells were spotted onto YPD-benomyl agar (70 μ g/ml) at the appropriate density and observed microscopically. In experiments utilizing cold-sensitive *tub2* strains, cells were spotted onto YPD agar at 11°C. For temperature-sensitive *cdc* strains, YPD agar at 37°C was used.

Staining of Cells for Microscopy and Flow Cytometry

Cells were fixed for microscopic observation with 70% ethanol and stained for DNA with 0.5 μ g/ml DAPI. Microtubule structures were observed using the anti-tubulin monoclonal antibody YOL1/34 and an FITC-conjugated secondary antibody as previously described (Stearns et al., 1990). For flow cytometric analysis, cells were fixed with 70% ethanol and stained for DNA with propidium iodide as described (Hutter and Eipel, 1978). For each sample, the DNA content of 10,000 cells was determined with a Coulter EPICS 752 flow cytometer.

Histone H1 Kinase Assay

Log-phase cells in YPD (pH 4.0) medium at an OD₆₀₀ of 0.15–0.2 were arrested for 4 hr with 8 μ g/ml α factor. Cells were then washed once and resuspended in the same medium without α factor, but with nocodazole as indicated. The cultures were incubated at 26°C and 1 ml aliquots removed at 15 min intervals. Cells were disrupted essentially as described (Langan et al., 1989). They were washed in cold SCE buffer and then resuspended in SCE plus 0.2 mg/ml 20T zymolyase (ICN). Cells were spheroplasted for 25 min at 30°C, washed in cold SCE, and resuspended in a cold solution of PK lysis buffer plus 1 mM PMSF. Extracts were centrifuged for 30 min at 145,000 \times g (4°C) and the supernatants adjusted to 120 μ g/ml (determined by the BCA assay [Pierce] using bovine serum albumin as the standard) with cold PK buffer plus PMSF.

Kinase reactions were performed at 24°C for 30 min with the protein extract at a final concentration of 40 μ g/ml, 1 mM DTT, 10 mM MgCl₂, 30 mM HEPES (pH 7.5), 10 mM ATP (pH 7.0), and 280 μ g/ml bovine histone H1 (Boehringer Mannheim) plus 50 μ Ci/ml [γ -³²P]ATP (final reaction volume was 18 μ l). Reactions were stopped with an equal volume of Laemmli sample buffer. Samples were subjected to electrophoresis in 11% polyacrylamide. Dried gels were analyzed with the Molecular Dynamics Phosphorimager, and the relative radioactivity of the histone H1 bands was quantified using Molecular Dynamics Imagequant software. The kinase activities measured at 0 hr after release from α factor arrest for any two strains differed by less than 15%.

Assay for Cytokinesis

Cells were synchronized with α factor and released into liquid medium containing nocodazole for 6 hr at 26°C. Formaldehyde was added to a final concentration of 5%, and cells were allowed to fix for 2 hr at room temperature. The cells were washed and resuspended in water. Glusulase (Du Pont) was added to 3% to half of each sample (Hartwell, 1971); the other half received no addition. Samples were rocked at room temperature for 2 hr, washed, and analyzed by microscopy.

DNA Manipulations

A *URA3-CEN* plasmid library of *S. cerevisiae* DNA (Rose et al., 1987) was transformed into either *bub1-1* or *bub2-1* strains. Ura⁺ transformants were replica transferred to YPD plates containing 7.5–15 μ g/ml benomyl. Resistant clones were then screened for loss of the *bub* continued-budding-in-benomyl phenotype (see above). Suppressing plasmids (seven for *bub1-1* and one for *bub2-1*) were extracted and transformed into *Escherichia coli* for analysis. Subcloning of the original genomic inserts to establish the boundaries of the genes was by standard procedures.

The cloned inserts could either represent the actual *bub* loci or suppressor loci. To distinguish between these possibilities, regions of

each genomic insert were subcloned into yeast integrating vectors. For the *bub2-1*-suppressing DNA, a 3375 bp BamHI fragment (Figure 6A) was inserted into the *URA3*-containing vector pRS308 (Sikorski and Hieter, 1989). This construct was cut with NheI and transformed into MAY591. The resulting strain was mated to the *bub2-1* strain, MAY1575. When sporulated, all 15 tetrads analyzed were of the parental ditype configuration (two *URA⁺*, *BUB⁺* spores and two *ura⁻*, *bub⁻* spores). This indicates that the cloned DNA is linked to the site of the *BUB2* locus.

For the *bub1-1*-suppressing DNA, a 2730 bp, EcoRI to HindIII fragment (Figure 6B) was inserted into pRS308. This construct was cut with BglII and transformed into MAY591. The resulting strain was mated to the *bub1-1* strain, MAY1726. When sporulated, this diploid gave rise to three parental ditype, six nonparental ditype, and eight tetratype asci. Therefore, this cloned DNA does not contain the *BUB1* locus, but instead encodes an unlinked suppressing locus. A similar result was obtained in a cross between a marked disruption of the suppressing locus (*bub3::LEU2*) and a *bub1-1* strain.

Marked disruptions of the two cloned *BUB* loci were generated by the one-step gene replacement method (Rothstein, 1983). For *bub2::URA3*, a DNA fragment containing *URA3* was inserted between the BamHI and HindIII sites in *BUB2*. For *bub3::LEU2*, a DNA fragment containing *LEU2* was inserted between the BglII and EcoRV sites in *BUB3*. Linear DNA from these constructs was initially used to transform the diploid strain MAY1210. The resulting transformants were sporulated to generate haploid null spores.

DNA sequencing was accomplished by subcloning into M13-based vectors and use of the Sequenase reagent kit (US Biochemical Corp.). All reported sequence was determined from both strands of DNA.

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GenBank Accession Numbers

The accession numbers are M84706 for *BUB2* and M64707 for *BUB3*.

SEELEY
DECLARATION
EXHIBIT NO. 22

The *Saccharomyces cerevisiae* Checkpoint Gene *BUB1* Encodes a Novel Protein Kinase

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Normal cell multiplication requires that the events of mitosis occur in a carefully ordered fashion. Cells employ checkpoints to prevent cycle progression until some prerequisite step has been completed. To explore the mechanisms of checkpoint enforcement, we previously screened for mutants of *Saccharomyces cerevisiae* which are unable to recover from a transient treatment with a benzimidazole-related microtubule inhibitor because they fail to inhibit subsequent cell cycle steps. Two of the identified genes, *BUB2* and *BUB3*, have been cloned and described (M. A. Hoyt, L. Totis, and B. T. Roberts, Cell 66:507-517, 1991). Here we present the characterization of the *BUB1* gene and its product. Genetic evidence was obtained suggesting that Bub1 and Bub3 are mutually dependent for function, and immunoprecipitation experiments demonstrated a physical association between the two. Sequence analysis of *BUB1* revealed a domain with similarity to protein kinases. In vitro experiments confirmed that Bub1 possesses kinase activity; Bub1 was able to autophosphorylate and to catalyze phosphorylation of Bub3. In addition, overproduced Bub1 was found to localize to the cell nucleus.

The production of two viable and equivalent daughter cells in mitosis requires that the events leading to cell division proceed in a carefully ordered fashion. Among other tightly regulated events, replicated sister chromosomes must be properly segregated, one to each daughter cell. For this reason, mitosis cannot be allowed to proceed if the genome has not been fully replicated or if chromosomes are not properly attached to a fully assembled mitotic spindle. Mechanisms required for ensuring the dependency of cell division on completion of such prerequisite steps have been termed checkpoints (12). The first checkpoint-associated gene product described was Rad9 (37), which is required for delaying the cell cycle prior to mitosis for repair of damaged DNA. However, the biochemical activity of Rad9 and its precise role in enforcing normal cell cycle dependency relationships are still unclear.

In most eukaryotic cells, disruption of the spindle results in a cell cycle delay until the spindle is reassembled (5). Treatment of wild-type yeast cells with benzimidazole-related microtubule inhibitors leads to cell cycle arrest in mitosis. Each treated cell has a large bud and replicated DNA, and the cyclical kinase activity of M-phase promoting factor (MPF) is maintained in a highly elevated state (14, 19, 20). Inactivation of MPF and progression into subsequent phases of the cell cycle are dependent on the reassembly of microtubules.

We have previously described two mutants of the yeast *Saccharomyces cerevisiae*, designated *bub1* and *bub2* mutants, which are unable to delay the cell cycle in response to loss of microtubules (14). A third gene, *BUB3*, was identified as an extra-copy suppressor of the *bub1-1* mutant. Like the *bub1* and *bub2* mutants, *bub3* deletion strains (*bub3Δ*) do not arrest at G₂/M when treated with large doses of benzimidazole but instead continue to bud and rapidly lose viability. Three mutations with similar phenotypes, designated *mad1*, *mad2*,

and *mad3*, have been described elsewhere (20). None of these are allelic to the *bub* mutations (11, 13a).

BUB2 and *BUB3* have been cloned and sequenced (14). Bub2 is closely related to the *cdc16⁺* gene product of *Schizosaccharomyces pombe* (7). The primary sequence of Bub3 is unrelated to sequences of other known proteins (14). Neither gene is essential, although *bub3* mutants grow very slowly. Here we present the cloning and characterization of *BUB1*, which encodes a gene product with a carboxyl-terminal domain similar to those of protein kinases. We demonstrate that Bub1 autophosphorylates in vitro and provide genetic and immunological evidence that Bub3 physically interacts with and is a substrate for the Bub1 kinase. We show that when overexpressed together, these proteins interfere with colony formation. Finally, we demonstrate that overproduced Bub1 is directed to the nucleus.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in these experiments were derivatives of S288C and are listed in Table 1. The screen which yielded the *bub1-1* mutation has been described, along with the construction of the *bub3Δ::LEU2* allele (14). Rich (YPD), minimal (SD), and sporulation media were as described elsewhere (30). The benzimidazole compound benomyl (DuPont) was added to solid media from a 10-mg/ml stock in dimethyl sulfoxide. Concentrations to assess benomyl sensitivity were in the range of 5 to 15 μg/ml. For overexpression in galactose liquid medium, strains were transferred from fresh patches on SD lacking uracil to liquid synthetic medium lacking uracil with 2% raffinose (Sigma) as the sole carbon source. After 24 to 36 h at 26°C, rapidly growing cultures were diluted to an optical density at 600 nm of 0.03, and galactose (Sigma G-0750) was added to 2%. Cultures were grown 12 to 14 h longer and then harvested. For galactose-induced overexpression on plates, fresh patches of strains on SD plates were transferred to solid synthetic medium lacking uracil with raffinose at 2% and grown for 30 h at 30°C. These strains were then transferred to synthetic agar medium with 2% galactose with various concentrations of benomyl and photographed after 3 days at 26°C. To examine

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TABLE 1. Yeast strains used

Strain	Relevant genotype
MAY589	<i>MATa BUB⁺ ura3-52</i>
MAY591	<i>MATa BUB⁺ ura3-52</i>
MAY1210	<i>MATa/MATa BUB⁺/BUB⁺</i>
MAY1800	<i>MATa bub1-1 ura3-52</i> (pMA1145 = <i>BUB3, URA3, CEN</i>)
MAY2072	<i>MATa bub3Δ::LEU2 ura3-52</i>
MAY2150	<i>MATa bub1-1 trp1-Δ1</i>
MAY2352	<i>MATa bub1-1 trp1-Δ1</i> (pTR37 = <i>BUB1, TRP1, CEN</i>)
MAY2409	<i>MATa BUB1::URA3::BUB1</i>
MAY2517	<i>MATa/MATa bub1Δ::HIS3/BUB1</i>
MAY2589	<i>MATa bub1Δ::HIS3</i>
MAY2740	<i>MATa/MATa bub1Δ::HIS3/BUB1 bub3Δ::LEU2/BUB3</i>
MAY3188	<i>MATa BUB⁺ ura3-52</i> (pTR144 = pGAL- <i>BUB1</i> , pGAL- <i>BUB3, URA3</i> , 2μm)
MAY3275	<i>MATa BUB⁺ ura3-52</i> (pTR147 = pGAL- <i>bub1K733R</i> , pGAL- <i>BUB3, URA3</i> , 2μm)
MAY3316	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR143 = pGAL- <i>BUB1, URA3</i> , 2μm)
MAY3317	<i>MATa BUB⁺ ura3-52</i> (YE352 = <i>URA3</i> , 2μm)
MAY3335	<i>MATa BUB⁺ ura3-52</i> (pTR143 = pGAL- <i>BUB1, URA3</i> , 2μm)
MAY3336	<i>MATa/MATa bub1Δ::URA3/BUB1</i>
MAY3337	<i>MATa bub1Δ::HIS3 ura3-52</i> (YE352 = <i>URA3</i> , 2μm)
MAY3339	<i>MATa bub1Δ::HIS3 ura3-52</i> (pTR150 = pGAL- <i>BUB3, URA3</i> , 2μm)
MAY3340	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR150 = pGAL- <i>BUB3, URA3</i> , 2μm)
MAY3341	<i>MATa BUB⁺ ura3-52</i> (pTR150 = pGAL- <i>BUB3, URA3</i> , 2μm)
MAY3343	<i>MATa bub3Δ::LEU2 ura3-52</i> (YE352 = <i>URA3</i> , 2μm)
MAY3419	<i>MATa bub1-1 ura3-52</i> (YE352 = <i>URA3</i> , 2μm)
MAY3420	<i>MATa bub1Δ::HIS3 ura3-52</i> (pMA1145 = <i>BUB3, URA3, CEN</i>)
MAY3421	<i>MATa bub1-1 ura3-52</i> (pTR143 = pGAL- <i>BUB1, URA3</i> , 2μm)
MAY3422	<i>MATa bub1-1 ura3-52</i> (pTR148 = pGAL- <i>bub1K733R, URA3</i> , 2μm)
MAY3423	<i>MATa bub3Δ::LEU2 ura3-52</i> (pTR148 = pGAL- <i>bub1K733R, URA3</i> , 2μm)
MAY3594	<i>MATa bub1Δ::URA3</i>
MAY3598	<i>MATa bub3Δ::LEU2</i>
DK4795-411	<i>MATa ade3</i>

inhibition of budding by microtubule disruption, *MATa* cells were arrested in G₁ with α-factor, released onto benomyl (70 μg/ml)-containing solid medium for 6 h, and observed microscopically as described previously (14).

DNA manipulations. A *TRP1-CEN* plasmid library of *S. cerevisiae* DNA (4a) was transformed into a *bub1-1 trp1-Δ1* strain (MAY2150). Trp⁺ transformants were replica transferred to YPD plates containing 10 μg of benomyl per ml. Resistant clones were then screened for loss of the *bub* continued-budding-in-benomyl phenotype as described previously (14). Plasmids from benomyl-resistant (Ben^R) colonies were extracted and transformed into *Escherichia coli* for analysis. Subcloning of the original genomic inserts to establish the boundaries of the genes was done by standard procedures. The cloned inserts could represent either the actual *BUB1* locus or a suppressor locus. To distinguish between these possibilities, the *Clal-SacI* region of the genomic insert from pTR37 was subcloned into the yeast integrating vector pRS306 to generate pTR50. This construct was cut with *XbaI* and transformed into MAY591. The resulting strain, which was *BUB⁺*, was mated to the *bub1-1* strain, MAY2150. When sporulated, this diploid gave rise to 16 parental ditype asci and no nonparental or tetratype asci.

Marked disruptions of *BUB1* were generated by the one-step gene replacement method (28). A 2.5-kb DNA fragment containing *HIS3* was inserted between the *XbaI* and *SmaI* sites in *BUB1* in pTR50, resulting in the deletion of 942 bp of the wild-type gene in pTR65. A 6.7-kb *EcoRV-SacI* fragment from this plasmid encompassing the deletion was used to transform the diploid strain MAY1210, yielding the *bub1Δ::HIS3* heterozygote MAY2517. The resulting transformants were sporulated to generate haploid null spores. Proper insertion was confirmed by Southern analysis using a radioactively labeled

2.6-kb *HpaI-PvuII* fragment of *BUB1* (data not shown). This same *BUB1* probe hybridized to yeast genomic DNA lambda library clone 4572 (data not shown) derived from chromosome VII (27a). Viability of putative *bub1* null mutants was later confirmed by creation of a more complete deletion of *BUB1*. A 2.5-kb fragment between the outermost *HindIII* sites, including all of the sequence with significant homology to known protein kinases, was replaced with *URA3* to make pTR92. A diploid strain heterozygous for *bub1Δ::URA3* (MAY3336) was generated by linearizing pTR92 with *SalI* and *BamHI* and transforming MAY1210 as described above. Survival and benomyl sensitivity of spores from the *bub1Δ::URA3* and *bub1Δ::HIS3* heterozygotes appeared identical.

Viability of *bub1Δ::HIS3 bub3Δ::LEU2* double-disruption mutants was established by transformation of the *bub3Δ::LEU2/BUB3* diploid strain MAY2067 (14) with *bub1Δ::HIS3* as described above to make MAY2740, a double heterozygote. This diploid was sporulated and analyzed as described above. Identical results were obtained by transforming MAY2517 (*bub1Δ::HIS3/BUB1*) with *StuI-SacI*-linearized pTR27 (*bub3Δ::LEU2*) (14) to yield MAY2739.

pTR168, a *CEN* plasmid marked with *LEU2* and that expresses *BUB1* from its normal promoter, was constructed by subcloning the *SalI-BamHI* fragment from pTR37 into pRS 315 (31). Overexpression of *BUB1* under the control of a galactose-inducible promoter was accomplished by inserting *BamHI* linkers at the *HpaI* site 24 bp 5' of the *BUB1* start codon and then transferring the resulting 3.6-kb *BamHI* fragment to the *BamHI* site of pBM272 (a gift from M. Johnston), yielding pTR134. A 4.6-kb *SalI-EcoRI* fragment from pTR134 including *BUB1* under the control of the *GAL1* promoter plus the linked *GAL10* promoter was transferred to the *URA3*-2μm plasmid YE352 (13), generating pTR143. *EcoRI* linkers were

ligated to a 1.1-kb *EcoRV* fragment beginning 31 bp upstream of the start ATG and ending 1 bp downstream of the stop TGA of *BUB3*. This fragment was inserted in the proper orientation for expression from the *GAL10* promoter at the *EcoRI* site of pTR143; this arrangement yields pTR144, a plasmid which overexpresses both proteins when grown in galactose medium. Overproduction of Bub3 alone was achieved by eliminating the *BUB1*-containing *BamHI* fragment from pTR144 to yield pTR150.

Construction of the *bub1K733R* allele was accomplished by site-directed mutagenesis (16, 17), using the synthetic oligonucleotide TO862 (GGACATCTGCGAGCGTTGAGAGTAG AAAAACCTGCAACT) as a primer. A small (251-bp) *PacI*-*BstI* 1071 fragment of the resulting allele, determined by DNA sequencing analysis to have only the single base pair substitution responsible for the *bub1K733R* mutation, was subcloned into the wild-type *BUB1* gene for expression. pTR170 is a *CEN* plasmid that expresses this mutant *BUB1* from its normal promoter and is otherwise completely identical to pTR168 containing wild-type *BUB1* (see above). Overexpression of *bub1K733R* along with *BUB3* was accomplished by replacing 251 bp encoding part of the presumed Bub1 ATP binding site in pTR144 with the mutated region described above, generating pTR147.

DNA sequencing was performed with the Sequenase reagent kit (United States Biochemical) following subcloning into M13-based vectors or by using double-stranded templates. All reported sequence was determined from both strands of DNA.

Production of antibodies. The 1.4-kb portion of *BUB1* between the two amino-terminal *HindIII* sites was expressed in *E. coli* BL21 from the pRSETC vector (Invitrogen) as pTR124. This arrangement adds 47 amino acids at the amino terminus, including a six-adjacent-histidine motif, and 20 amino acids at the carboxyl terminus. A 200-ml culture in log phase was induced to express the fusion protein with 1 mM isopropylthiogalactopyranoside (IPTG) and then harvested. Cells were lysed by heavy sonication in buffer A (100 mM sodium phosphate, 10 mM Tris, 8 M urea [pH 8.0]). After spinning out insoluble debris, this sample was loaded three times over Ni^{2+} -nitrilotriacetic acid-agarose (Qiagen). The column was washed with 3 volumes of buffer A and 3 volumes of buffer B (buffer A, pH to 6.3). Finally, samples were eluted with buffer C (buffer A, pH to 5.9). Fractions containing the largest amounts of the fusion protein were pooled and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). The major band, with an apparent molecular mass of 64 kDa as visualized by copper chloride staining, was excised and then pulverized. Immunizations of two New Zealand White rabbits, designated 1026 and 1029, were performed by HRP Inc. (Denver, Pa.) by standard methods. Serum from rabbit 1026 was used in all experiments described herein because it had a higher affinity for Bub1 than that from rabbit 1029. Serum from the fourth bleed of this rabbit, designated 1026-4, was used in these experiments. Where noted, these antibodies were purified by the method of Olmsted (21) as described by Pringle et al. (27), using the 64-kDa fusion protein from pTR124 described above. Whole cell lysate from a 500-ml culture of cells expressing the 64-kDa protein was separated by preparative SDS-PAGE for transfer to nitrocellulose. The major band as visualized by Ponceau S staining was excised and treated exactly as described previously (27). Following elution and neutralization, antibodies were concentrated to approximately serum concentration in phosphate-buffered saline (PBS), using a Centricon (Amicon) spin filter system (molecular mass cutoff of 10^4 kDa).

Bub3-specific antibodies were a generous gift from Suzanne Guénette and Frank Solomon.

Immunofluorescence. Cells overexpressing *BUB* genes as described above were sonicated briefly and then fixed for 2 h in 3.7% formaldehyde at room temperature. Fixed cells were prepared for immunofluorescence microscopy as described previously (27) and incubated overnight at 4°C in 1:50-diluted affinity-pure 1026-4 primary antibodies (described above) and 1 h at room temperature with 1:250-diluted fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies.

Immunoprecipitation and autophosphorylation analysis. Immunoprecipitations were performed with affinity-purified antibodies from the fourth bleed of rabbit 1026 (1026-4) or preimmune antibodies from the same rabbit subjected to the same affinity purification regimen. In each case, a quantity of affinity-pure antibodies corresponding to 20 μl of untreated serum was used to immunoprecipitate Bub1 from 300 to 360 μg of soluble yeast protein. These protein extracts were obtained from strains described above by bead beating in the cold with PBS plus a freshly added protease inhibitor cocktail including leupeptin (0.5 $\mu\text{g}/\text{ml}$), chymostatin (0.1 $\mu\text{g}/\text{ml}$), aprotinin (2 $\mu\text{g}/\text{ml}$), and antipain (2.5 $\mu\text{g}/\text{ml}$) from a 100 \times stock in water, plus pepstatin A (1 $\mu\text{g}/\text{ml}$) and phenylmethylsulfonyl fluoride (10 $\mu\text{g}/\text{ml}$) from a 100 \times stock in ethanol. Antibody incubations were performed at 4°C for 3 to 5 h in PBS plus protease inhibitors (PBSPI). Immune complexes were collected by incubation at 4°C for 0.5 to 1.5 h with 80 μl of 50% protein A-Sepharose (Sigma P-3391) in PBS plus 0.1% bovine serum albumin (BSA) plus protease inhibitors (PBSBPI). Immune complexes were washed at least six times with 10 volumes of PBSBPI. An aliquot was washed at least two additional times with 10 volumes of PBSPI (no BSA) and, after boiling with 2 \times sample buffer, reserved for Western blot (immunoblot) analysis. The remainder was treated essentially as described previously (4). Briefly, immune complexes were washed twice in 10 volumes of autophosphorylation buffer (50 mM Tris [pH 7.5], 10 mM MgCl_2 , 1% Triton X-100 [Sigma]). The volume was reduced to 30 μl , and 30 μCi of [γ - ^{32}P]ATP (Amersham) was added to each. The reaction was allowed to proceed 15 min at room temperature and was then terminated by addition of 2 \times sample buffer and boiling. After SDS-PAGE, Coomassie blue staining, and destaining in 10% methanol-7% acetic acid-10 mM sodium pyrophosphate, radioactive bands were identified by autoradiography.

Western transfers to nitrocellulose (Schleicher & Schuell) to assess *BUB1* and *BUB3* overexpression and efficiency of immunoprecipitation were performed by standard methods (36). Proteins were visualized by using the Western Light (Tropix) chemiluminescence detection kit, with the modification that 5% powdered milk was added to the blocking agent, and blots were blocked overnight at 4°C. Except where noted, Western blots were probed with unpurified antiserum diluted 500- to 1,000-fold and incubated for 1 to 2 h at room temperature or overnight at 4°C.

Nucleotide sequence accession number. The sequence of the *BUB1* gene has been submitted to GenBank and assigned accession number L32027.

RESULTS

Cloning of *BUB1*. *BUB1* was cloned by transformation of the *bub1-1* strain MAY2150 with a yeast genomic library. Transformants were replica transferred to media containing 10 μg of benomyl (a benzimidazole compound) per ml, and resistant colonies were selected. Plasmids from the Ben^R colonies were isolated, retransformed into the original *bub1-1* strain, and

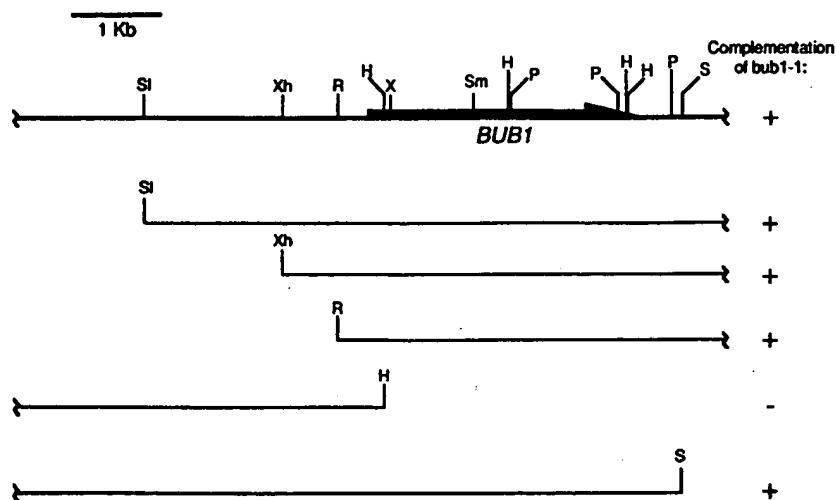


FIG. 1. Restriction enzyme site map of the *BUB1* locus. Thickened regions of the map indicate the extent of the open reading frame, with the arrowhead directed toward the 3' end. Lines beneath the restriction maps indicate DNA that is contained within tested subclones. + or - indicates whether the subclone rescues *bub1-1*. Restriction enzyme sites: SI, *SalI*; Xh, *XhoI*; R, *EcoRI*; H, *HindIII*; X, *XbaI*; Sm, *SmaI*; P, *PstI*; S, *SacI*.

then tested again for ability to restore wild-type benomyl resistance. Those which invariably yielded Ben^R colonies were selected for further study.

Since *BUB3* is an unlinked, extra-copy suppressor of *bub1-1* (14) and extra copies of α -tubulin genes have been shown to increase benomyl resistance in wild-type cells (29), the restriction enzyme maps of genomic clones obtained by the screen were compared with the maps of *BUB3* and the yeast α -tubulin genes, *TUB1* and *TUB3*. One plasmid, pTR37, had a distinct restriction site pattern. The cloned region was marked with *URA3*, using a yeast integrating vector in a *ura3-52 BUB*⁺ haploid strain. The resulting strain, which was also *BUB*⁺, was mated to a *ura3-52 bub1-1* strain and sporulated. The spores were scored for benomyl resistance and uracil prototrophy. All 16 tetrads tested were parental ditypes with respect to these phenotypes (2 Ura⁺ Ben^R spores: 2 Ura⁻ Ben^S spores). Among three tested tetrads, all Ura⁻ spores continued to bud on high concentrations of benomyl, and all Ura⁺ spores arrested normally. Taken together, these results indicate a tight linkage between *bub1-1* (≤ 8 centimorgans) and the introduced *URA3* marker and suggest that pTR37 includes the wild-type *BUB1* gene.

A radioactively labeled fragment of the *BUB1* gene was used to probe a Southern blot of *S. cerevisiae* chromosomes separated by pulsed-field gel electrophoresis. This experiment indicated that *BUB1* is located on chromosome VII. Hybridization of the same fragment to a lambda library of yeast genomic DNA fragments demonstrated linkage to *ADE3* (see Materials and Methods). This linkage was confirmed by tetrad analysis of a diploid heterozygous for *ade3* and a *URA3*-marked allele of *BUB1*. This experiment indicated that *BUB1* and *ADE3* are located approximately 12 centimorgans apart (39 parental ditype: 0 nonparental ditype: 12 tetratype).

Disruption of *BUB1*. Subcloning of DNA from pTR37 indicated that rescue of the *bub1-1* phenotype was conferred by a region contained between the *EcoRI* and *SacI* sites located 3.7 kb apart (Fig. 1). This region was sequenced and shown to contain a single 3,063-bp open reading frame (Fig. 2). Marked disruptions of *BUB1* were generated by the one-step gene replacement method (28) (see Materials and Methods).

bub1::HIS3 deleted DNA between the *XbaI* and *SmaI* sites internal to the *BUB1* gene (Fig. 1). *bub1 Δ ::URA3* deleted all of the DNA between the outermost *HindIII* sites in the *BUB1* gene, causing elimination of almost the entire open reading frame, including all sequences encoding protein kinase similarities (see below). Both *BUB1* disruptions caused identical phenotypes. Transformation of a wild-type diploid strain with these constructs yielded *bub1 Δ /BUB1* heterozygotes (MAY 2517 and MAY3336). When sporulated, the *bub1 Δ* spores germinated with reduced efficiency (Fig. 3); for MAY3336, 66% (76 of 116) of Ura⁺ spores failed to form colonies, while only 4% (5 of 116) of Ura⁻ spores failed. The viable *bub1 Δ* spores formed colonies that grew at much slower rates than their *BUB1* sisters (Fig. 3). When propagated, this slow-growth phenotype partially reverted, probably reflecting the accumulation of suppressing mutations. *bub1 Δ* cells were also supersensitive to benomyl (dead at 7.5 μ g/ml, compared with 15 μ g/ml for the wild type; see Fig. 5). Similar phenotypes were reported for *bub3 Δ* mutants (14) although *bub3 Δ* spores germinated with wild-type efficiency.

bub deletion mutant cells were examined for the ability to halt their budding cycles when microtubule function was compromised (Table 2). Mutant and wild-type cells were synchronized in G₁ with α -factor and released onto medium containing a high concentration of benomyl. After 6 h, the *BUB*⁺ cells had stopped budding and arrested with a large-budded morphology (equivalent to two cell bodies in Table 2). In contrast, like the *bub1-1* mutant (14), both the *bub1 Δ* and *bub3 Δ* mutants frequently produced extra buds in the presence of benomyl.

The predicted *BUB1* gene product has similarity to known protein kinases. The predicted 1,021-amino-acid, 118-kDa protein encoded by the *BUB1* gene (Fig. 2) was compared with proteins in the GenPept database, using the FASTA and BLAST programs (1, 25). A carboxyl-terminal portion displays significant similarity to known serine-threonine protein kinases (Fig. 4). In particular, Bub1 has strong homology to kinases in the regions designated I, II, III, VI, and VII and weak homology in region IX (10). Note that the highly conserved regions VIII and XI are not found in Bub1, and there is an

-100 TACTACACAATATACGTGATTTTGAATAGAGTATAAAGTTTTCATCATGGAAAGATTATTGACGGTTCCTATTGTTTGAATGTTAAACGCTGACCAGGAA
 1 M N L D L G S T V R G Y E S D K D T F P Q S K G V S S S Q K E Q H S
 1 ATGAATTTAGATTAGGTTCTACTGTACGAGGCTATGAATCAGATTAAGACACATTTCCGCAATCGAAAGGCGTTAGCTCATCTCAAAAAGACGACGATA
 35 Q L N P Q T K I A Y E Q R L L N D L E D M D D P L D L F L D Y M I W
 101 GTCACTGAATCAGACAAAATAGCTTATGAACAGAGACTTCTAAATGATTAGAGGATATGGATGATCCTTAGATTGTTTGTATATGATATG
 68 I S T S Y I E V D S E S G Q E V L R S T M E R C L I Y I Q D M E T
 201 GATTAGCACCAGCTATATTGAAGTAGATTCTGAATCAGGTCAAGAGTTCTCCGAAGTACAATGGAAGATGCTTGATTTACATACAGATATGGAACCC
 101 Y R N D P R F L K I W I W Y I N L F L S N N F H E S E N T F K Y M F
 301 TATAGGAACGATCCGAGATTTTAAAAATTTGGATTGGTATATAAATTTGTTCTTAGCAACAACCTCCACGAGAGTGAATACTTTTAAATATATGT
 135 N K G I G T K L S L F Y E E F S K L L E N A Q F F L E A K V L L E
 401 TTAACAAGGGAATCGGAACAAAGCTTTCGTTATTTTATGAAGAGTTCTCCAAGCTACTAGAAAATGCTCAATTTTTCTAGAACGAAGGTGTTATTAGA
 168 L G A E N N C R P Y N R L L R S L S N Y E D R L R E M N I V E N Q
 501 ACTAGGGGCTGAAAACAAATTCGAGGCCCTTACACAGACTATTAAAGTCATTAAAGCAATATTAAGACAGACTCCGAGAAATGAACCTGTAAGAAACAG
 201 N S V P L K N D S R E R L K G R L I Y R T A P F F I R K F L T S S L M T D
 601 AACAGTGTACCTGATTCAAGGGAAGATTAAGGGAAGGTTAATTTATAGAACAGCACCATTTCATTTCGTAATTTTAAACCTCATCTGATGACTG
 235 D K E N R A N L N S N V G V G K S A P N V Y Q D S I V V A D F K S
 701 ATGACAAAGGAAATCGTGGCAACCTGAATCTAATGTTGGAGTGGTAAGTCTGCTTAAAGTATATCAAGATTCTATAGTGGTGGTGAATTTAAAGTC
 268 E T E R L N L N S S K Q P S N Q R L K N G N K K T S I Y A D Q K Q
 801 AGAAACAGAGAGGCTTAATTTGAATAGCTCTAAACAGCCTCTAACCAAGCCTTGAAGATGGAATAAAAAAACTCAATATATGCGATCAAAAAGCAA
 301 S N N P V Y K L I N T P G R K P E R I V F N F N L I Y P E N D E F
 901 TCTAACATCCGACTTACAACTAATCAATCTCGGGACGGAACCTGAACGATAGTTTTTAATTTAATTAATATCCGAAATGATGAGGAGT
 335 N T E E I L A M I K G L Y K V Q R R G K K H T E D Y T S D K N R K
 1001 TCAACACCGAAGAAATTTGGCAATGATTAAAGGACTTACAAAGTACAGCGCAGAGGTAAGAAGCACACCGAAGATTATACAAAGTCAAAAAATAGGAA
 368 K R K L R Q D L V L E R R Q D L P S S Q P P V P K S T R I E V F K D
 1101 GAAACGTAAGTCTGCTGCTGGTAGAGAGCGGAAGACTTACCTTACAGCCACCGCTTGTCCCAAACTACGCGAATGAGGTGTTTAAAGAT
 401 D D N P S Q S T H H K N T Q V Q V Q T T T S I L P L K P V V D G N L
 1201 GAGGATATCCAGCCAGGACACACCAATAAAAAATACACAGGTACAAGTCAAGCAACGCTCTATTTACCTTTGAAGCCTTGCTGGTGTGATGAAACCC
 435 A H E T P V K P S L T S N A S R S P T V T A F S K D A I N E V F S
 1301 TCGCGCATGAGACACAGTGAAGCCCTCACTAATCAACGCTTCCCGTTACCTACAGTAAACAGCTTTTCAAAAGAGCGCTATAAATAGGCTTTTTC
 468 M F N Q H Y S T P G A L L D G D T T T S K F N V F E N Q E F
 1401 CATGTTCAATCAATATTCAACCCCGGCTGCGCTATTAGATGGTATGATACCACTACAAGTAAATCAACGCTTCCGAAATTTTACCAGAAATTT
 501 T A K I E D L T E V K D P K Q E T V S Q Q T T S T N E T V R Y E
 1501 ACAGCAAGAATATTGAAGATTAACTGAAGTTAAAGATCTAAACAAGAGCGGTGTCACAACAACTACCTCAACAAATGAACCAACGTACGATATG
 535 R L S N S S T R P E K A D Y M T P I K E T T E T D V V P I I Q T P
 1601 AAAGATTGTCGAATAGTACACAGGCCAGAAAGGAGACTACATGACGCTTAAAGAAAGAACTACTGAAACAGATGGTGTACCTTAATCCAGACACC
 568 K E Q I R T E D K K S G D N T E T Q T Q L T S T T I Q S S P F L T
 1701 TAAGGAACAAATAAGGACAGAAAGATAAGAAATCGGGGATAATACAGAAACAAACTCAATTAACAAGCAACCACTTCAAAAGTTCTCCATTCTCACA
 601 Q P E P Q A E K L L Q T A E H S E K S K E H Y P T I I P P F T K I K
 1801 CAACCTGAACCAAGCAGAAAGCTTTTACAACTGCAAGCAATTCGGAAGAGTAAAGAGCATTACCAACGATCATACCTCCCTTCACTAAATAA
 635 N Q P P V I I E N P L S N N L R A K F L S E I S P P L F Q Y N T F
 1901 AAAATCAACCCGCTGCTATTATTGAACCCACTTAGTAATAATTAAAGAGGAAGTTTTATCCGAAATTTCCGCCCATTTTCCCAATATAACACCTT
 668 Y N Y N Q E L K M S S L L K K I H R V S R N E N K N P I V D F K K
 2001 TTATACTACACCAAGATTGAAATGAGTTCTCTATTGAAAAATCCATAGGGTATCCGCAACGAAACAAAGAACCAATTTGTTGACTTCAAGAG
 701 T G D L Y C I R G E L G E G Y A T V Y L A E S S Q G H L R A L K V
 2101 ACGGGTATTTATGTTATGATCGGAGAGATTAGGCGAAGGAGTTATGCCACTGTATATAGCAGAACTAGTCAAGGACATCTGGCAGGTTGAAAG
 735 E K P A S V W E Y I I E N P L S N N L R A K F L S E I S P P L F Q Y N T F
 2201 TAGAAAACTGCAAGTGTATGGGAATATTACATAATGAGCCAAGTGGAGTTAGGTTGAGGAAGACACAATATTAAAGTCAATATCAACGCTAGTGC
 768 L H L F L D E S Y L V L N Y A S Q G T V L D L I N L Q R E K A I D
 2301 TTTACACTTGTTCCTGTAGAGAGCTACCTTGTTTAAACTACGCGTCAAGTACGCTTGGATTAAATTAATTTACAAAGGTAAGGCAATTTGAC
 801 G N G I M D E Y L C M F I T V E L M K V L E K I H E V G I I H G D L
 2401 GGCAACGGAATAATGGATGAATTTTGTGATGTTTATCACTGCTGAGCTAATGAAGTACTTGAAGAGATACATGAAGTGGGAATAATACATGGCGATT
 835 K P D N C M I R L E K P G E P L G A H Y M R N G E D G W E N K G I
 2501 TGAACACAGATAACTGCATGATCAGATTAGAAAAACAGGCAACCCCTGGGTGCTCATTATATGCGCAATGGAGAGGATGGCTGGGAAACAAAGGGAAT
 868 Y L I D F G R S F D M T L L P P G T K F K S N W K A D Q Q D C W E
 2601 CTATTTAATAGATTTCGGTAGATCATTGATATGACACTGTACCACCTGGAAGCAAAATCAAAATCAAAATGGAAGGCTGATCAGCAAGATTTGCTGGGAA
 901 M R A G K P W S Y E A D Y Y G L A G V I H S M L F G K F I E T I Q L
 2701 ATCGTGGCGGCAAGCCATGGAGTATGAAGCGGATTATTATGGATTAGCAGCGCTACATCATCTGATGCTTTTGGAAATTCATAGAAACCAATCCAGC
 935 O N G R C K L K N P F K R Y W K K E I W G V I F D L L L N S G Q A
 2801 TGCAGATGGAGGATGCAATTTGAAGAACCCATTCAAAGGATTGGAAAAAGAAATATGGGCGTTATATTGATTACTGTTAAATAGCGGTCAAGC
 968 S N Q A L P M T K I V E I R N L I E S H L E Q H A E N H L R N V
 2901 TTCCAATCAAGCTTTCGGATGACTGAAAGATTGTTGAAATAGGAATTTGATAGAAAGCCATCTGGAACAGCAGCAGAAAAATCACTTAAGAAACGTA
 1001 I L S I E L S I E L S H F Q Y K G K P S R R F *** 1021
 3001 ATTCTAAGATTGAAGAGGAGTTATCAATTTTCAATTAAGGGGAACCGTCAAGGAGATTAGTGAATAAATATGCCTTCATCTCCCGCATAGGTGA

FIG. 2. DNA sequence of the *BUB1* gene. The amino acid sequence predicted by the *BUB1* open reading frame is indicated in single-letter code. Nucleotide position 0 is the A in the predicted initiation codon. Not shown is an additional 760 bp of 5' sequence determined. There are no reasonable initiation codon candidates in this 5' region, which also includes numerous termination codons in all three reading frames.

unusually large number of amino acids between regions VI and VII. Despite a detectable similarity to serine-threonine protein kinases, none in the current GenPept database (release 79.0) is particularly closely related. The most similar relative to Bub1 is PknA, a putative protein kinase from an *Anabaena* isolate (38), which is 27% identical over 209 amino acids. The amino-terminal 700-amino-acid portion of Bub1 is not significantly related to other gene products in the database but was found to be related to the amino terminus of the product of the recently sequenced *MAD3* mitotic checkpoint gene (11) (see Discussion).

An invariant lysine corresponding to residue 733 of Bub1 has been shown to be essential for catalytic activity in many protein kinases. By site-directed mutagenesis, we made a conservative mutation of this lysine to arginine, designated

bub1K733R. We constructed two *LEU2*-containing plasmids, pTR168 (*BUB1*) and pTR170 (*bub1K733R*), that differed only at the site of this mutation (see Materials and Methods). Both were transformed into the MAY3336 diploid (*bub1Δ::URA3/BUB1*), and the resulting strains were sporulated. For pTR168, all *Ura⁺ Leu⁺* spores exhibited wild-type growth rates and benomyl resistance, indicating that this construct could complement the *bub1Δ::URA3* phenotypes. In contrast, pTR170-containing *Ura⁺* spores were still slowly growing and benomyl supersensitive, indicating that the *bub1K733R* mutant could not complement. However, *bub1K733R* was able to partially suppress the benomyl-supersensitive phenotype of the *bub1-1* putative missense allele (Fig. 5).

Genetic evidence suggests that Bub1 and Bub3 interact. The similarity of the *bub1Δ* and *bub3Δ* phenotypes and the sup-

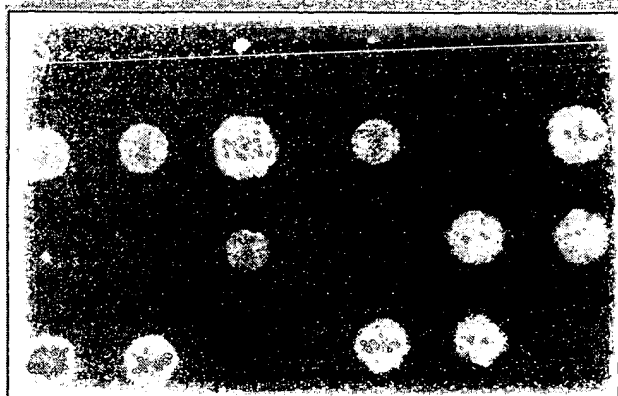


FIG. 3. Dissected tetrads from a *bub1Δ::URA3* heterozygote. Six tetrads from strain MAY3336 (*bub1Δ::URA3/BUB1+*) were dissected in vertical rows onto rich medium. Each tetrad contained two *Ura*⁻ (*BUB1+*) spores that grew into large colonies and two *Ura*⁺ (*bub1Δ::URA3*) spores that were either inviable or produced slowly growing colonies. The colony color differences are due to the segregating *ade2* mutation.

pression of *bub1-1* by extra *BUB3* suggested that the products of these genes might operate in the same functional pathway. For this reason, we examined the phenotype of *bub1Δ::HIS3 bub3Δ::LEU2* double mutants. Haploid double mutants were viable but slowly growing and supersensitive to benomyl, and their phenotype was no more severe than that of the single mutants. These data contribute to the conclusion that Bub1 and Bub3 are both integral components of the same nonessential system.

High-copy-number vectors bearing either *BUB1* or *BUB3* under control of the galactose-inducible *GAL1* and *GAL10* promoters, respectively, were constructed and transformed into wild-type yeast cells. Both resulting strains grew well on galactose, although strains overexpressing *BUB1* exhibited mild benomyl sensitivity (Fig. 5). However, overexpression of both genes in the same strain resulted in a severe growth defect. These strains did not accumulate in a uniform phase of the cell cycle, and the explanation for their growth defect was not clear. Interestingly, strains overexpressing *BUB3* plus the kinase-inactive *bub1K733R* mutant exhibited a phenotype similar to but slightly stronger than that of strains overexpressing the wild-type proteins, suggesting that overphosphorylation due to the putative kinase activity of Bub1 did not cause the growth defect.

Bub1 exhibits protein kinase activity *in vitro*. A common feature of many protein kinases is the ability to autophosphorylate. As a means of testing whether Bub1 is an authentic protein kinase, we examined the ability of Bub1 to autophos-

PKC GKSGFGKVLADRKGTTEE--LYAIRILKKDVIQDDVECTMVB
MLCK GRGAFSIVYLGENKQT--KQRYAIRVINKSELGK-DYEKNLKMZ
SAK QSGSGFGKVLADRKGTTEE--LYAIRILKKDVIQDDVECTMVB
mos GAGGFGSVYKATYRG-VP---VAIRQVKNCTKNRLASRRSFVAB
PknA GSGGFGSETFLAEDSQMPNRRCCVVKQLRP IHNHP-QIYQL-VQB
Bub1p 712 GEGGYATVYLAESSQGH--LR-ALRVEKPASVW--EYIMSQVB 750

I

II

III

PKC GIIYRDLLKDEHMLDSEG-----HIKIADFG
MLCK NIVHRDLKPEHLLKSKENHL-----EVAIDFG
SAK SLIYRDLLKPEHLLDHLG-----YLVKTFDFG
mos SIYHLDLKPAILISEQD-----VCKISDFG
PknA RIHRDLKPEHILRHRDG-----KPVLDIFG
Bub1p 828 GIHGDLLKPEHMLRLEKPEGLAHYMYNGEDGWENKGIYLDIFG
873

VI

VII

PKC PDYIAPBIIAYQPYGKSDVWYWG
MLCK PSYVAPFVLNATGYDKEVDMWSIG
SAK PQYLAPFVILNRSYASQWTTGGLG
mos YTHRAPFLLKGEVTPKADIYSFA
PknA PGYMPSEQAAGRPV YSSDLYSLG
Bub1p 894 DQDCWDRHAKKPNSEADYGLA 917

VIII

IX

FIG. 4. Limited comparison of primary structures of Bub1 and known protein kinases. Regions of the putative catalytic domain sequence of Bub1 are aligned with corresponding regions from known serine-threonine protein kinases. Boldface residues are those most conserved in serine-threonine protein kinases (10). PKC, protein kinase C; MLCK, myosin light-chain kinase; SAK, spermatozoan-associated kinase; mos, human *c-mos* product; PknA, putative protein kinase from *Anabaena* sp.

phorylate *in vitro*. A fusion protein including the predicted amino acids 141 to 609 of *BUB1* (amino terminal to the putative kinase domain) was expressed in *E. coli*, purified, and injected into rabbits. Affinity-purified polyclonal antisera from these rabbits specifically recognized a group of proteins with apparent molecular masses ranging from approximately 125 to 135 kDa and several smaller proteins (Fig. 6). The predicted molecular mass of Bub1 is 118 kDa. (In addition, a band of approximately 65 kDa was nonspecifically recognized by this affinity-pure preparation.) Note that these protein bands were evident only in extracts from cells overexpressing *BUB1* (either along with overexpressed *BUB3* as shown or in cells with wild-type Bub3 levels [not shown]), not from wild-type yeast cells, and were not recognized by preimmune serum (Fig. 6). These specific bands were also completely absent from cells expressing a *BUB1* gene truncated by removing the two 3' *Pst*I fragments (data not shown). Taken together, these observations indicate that these antibodies specifically recognize cellular forms of Bub1. The affinity-pure antibodies were used to immunoprecipitate Bub1 from extracts made from overproducing cells. These precipitates were Western blotted and probed with the same antibodies. Only bands corresponding to 125 and 130 kDa were observed, although the dense band caused by the precipitating antibody heavy chains might have obscured forms near 50 kDa (Fig. 7 and data not shown). It is likely that the other molecular mass forms of Bub1 visible in the Fig. 6 Western blot are proteolytic fragments or other short-lived forms.

These antibodies were used to immunoprecipitate overproduced Bub1 from the soluble portion of whole cell lysates. One-third of the immune complex sample was incubated in a buffer containing $MgCl_2$ and $[\gamma\text{-}^{32}P]ATP$ and then subjected to SDS-PAGE and autoradiography (Fig. 7). The remaining two-thirds were subjected to SDS-PAGE, then blotted to nitrocellulose, and probed with antibodies to Bub1. The autophos-

TABLE 2. Cell morphologies on benomyl-containing medium

Strain	Genotype	% of cells with indicated no. of cell bodies ^a			
		2	3	4	>4
MAY589	<i>BUB+</i>	89	9	2	0
MAY3594	<i>bub1Δ::URA3</i>	47	37	4	12
MAY3598	<i>bub3Δ::LEU2</i>	54	41	4	1

^a Values are percentages of total cells exhibiting the indicated number of cell bodies after release from α -factor arrest followed by 6 h on benomyl (70 μ g/ml)-containing solid medium. Immediately after release from α -factor, 88 to 94% of the cells had unbudded morphologies (one cell body).

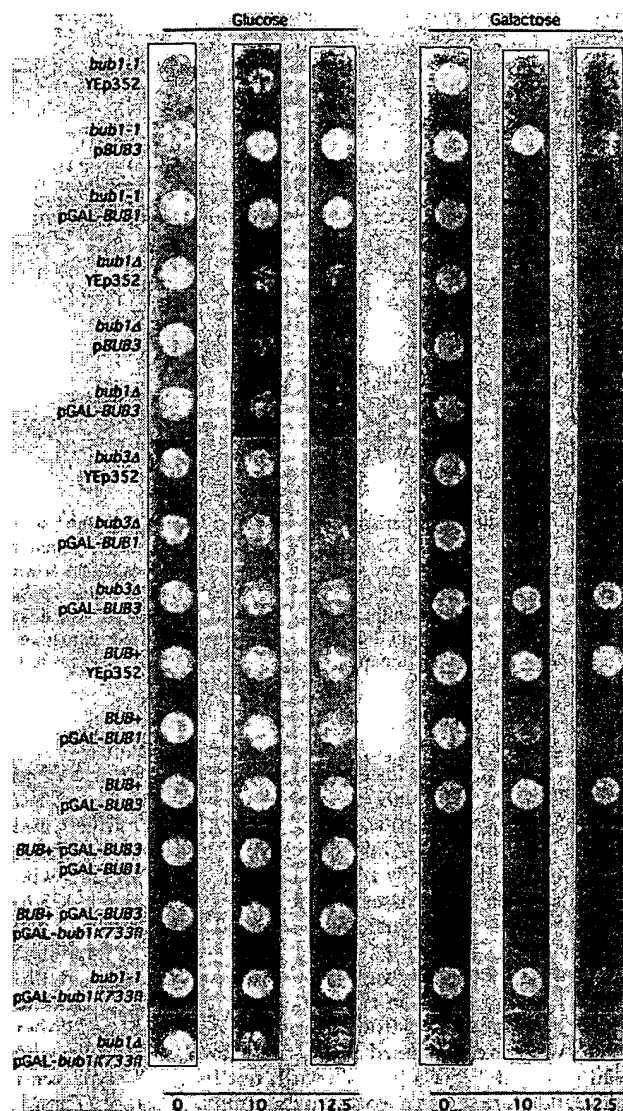


FIG. 5. Growth and rescue of *bub* mutants and strains overproducing Bub proteins. Strains were derepressed on synthetic media with raffinose and then plated to glucose or galactose medium containing the concentration of benomyl (in micrograms per milliliter) indicated at the bottom (see Materials and Methods for details of plasmid construction and growth conditions). For each horizontal row of cells, the chromosomal genotype is indicated followed by the relevant plasmid-borne genes. pGAL indicates that the following gene is expressed from a galactose-inducible promoter. Note that both galactose promoter-driven *BUB1* and *BUB3* express sufficient gene product on glucose medium to cause phenotypic consequences. All plasmids have the *URA3* marker. pBUB3 is a *CEN* vector, but all other plasmids are multicopy 2 μ m vectors derived from YEp352. Strains: *bub1-1* YEp352, MAY3419; *bub1-1* pBUB3, MAY1800; *bub1-1* pGAL-BUB1, MAY3421; *bub1Δ* YEp352, MAY3337; *bub1Δ* pBUB3, MAY3420; *bub1Δ* pGAL-BUB3, MAY3339; *bub3Δ* YEp352, MAY3343; *bub3Δ* pGAL-BUB1, MAY3316; *bub3Δ* pGAL-BUB3, MAY3340; *BUB*⁺ YEp352, MAY3317; *BUB*⁺ pGAL-BUB1, MAY3335; *BUB*⁺ pGAL-BUB3, MAY3341; *BUB*⁺ pGAL-BUB1 pGAL-BUB3, MAY3188; *BUB*⁺ pGAL-bub1K733R pGAL-BUB3, MAY3275; *bub1-1* pGAL-bub1K733R, MAY3422; *bub1Δ* pGAL-bub1K733R, MAY3423.

phorylation experiment revealed a major phosphate-labeled band of approximately 130 kDa. In the parallel immunological detection experiment, anti-Bub1 antibodies detect a doublet of proteins corresponding to molecular masses of 125 and 130 kDa. No bands of this size were observed in either experiment when preimmune serum was substituted for immune serum in the immunoprecipitation. Furthermore, none of these bands were observed unless *BUB1* was overexpressed in the cells from which the extracts were made. Therefore, we conclude that 130-kDa phosphoprotein and the immunologically detected doublet represent forms of the authentic *BUB1* gene product.

Since it was possible that the observed phosphorylation of Bub1 was catalyzed by a contaminating protein kinase, the experiment was also performed with the *bub1K733R* mutant described above. When the mutant allele was overexpressed, it produced a protein of the size of Bub1 that was visible by Western analysis. No significant incorporation of ³²P was observed for this protein (Fig. 7A, lane 4). Note that in some experiments with this mutant, a very faint band was observed at the molecular mass of Bub1 (data not shown). This weak signal may be due to the presence of a contaminating protein kinase in the immunoprecipitate or possible low residual activity of the mutant gene product. Although the lysine-to-arginine substitution is highly conservative, it appears to reduce the stability of the protein, and consequently a smaller amount mutant protein was immunoprecipitated from an equivalent amount of cell lysate. To compensate for this in the experiment, a portion of the wild-type Bub1 complexes was diluted fivefold and analyzed next to the *bub1K733R* mutant. Even in the diluted wild-type lane (Fig. 7A, lane 3), ³²P incorporation was much higher than for the *bub1K733R* mutant. These data indicate that Bub1 exhibits protein kinase activity in vitro.

Bub3 physically interacts with and is a substrate for the Bub1 protein kinase in vitro. Several smaller proteins, ranging from approximately 30 to 90 kDa, were also phosphate labeled in the Bub1 immunoprecipitates (Fig. 7A and 8A). These could represent other coimmunoprecipitating substrates, degraded forms of phosphorylated Bub1, or even phosphorylated antibody chains. Since Bub3 has a predicted molecular mass of 39 kDa, we tested the hypothesis that the 40-kDa protein prominently labeled in the precipitates represented the *BUB3* gene product. Although the 40-kDa protein band could sometimes be seen when *BUB3* was expressed from its chromosomal promoter, the band increased in intensity when *BUB3* was overexpressed along with *BUB1* (Fig. 8A). When the autophosphorylation assay was performed with extracts from *bub3* deletion mutants overexpressing *BUB1*, the 40-kDa band was absent. Furthermore, a protein of the same molecular mass was recognized by Bub3 antibodies in Bub1 immunoprecipitates (Fig. 7B and 8B). We conclude from these observations that Bub1 and Bub3 coimmunoprecipitate and that the *BUB3* gene product is an in vitro substrate for Bub1.

Overexpression of *BUB3* along with *BUB1* resulted in a consistently higher level of labeling of all proteins phosphorylated in the experiment compared with overproduction of Bub1 alone (Fig. 8A, lane 1 versus lane 2). Although overproduction of Bub3 tended to boost the amount of Bub1 immunoprecipitated in some trials (data not shown), in this experiment a comparable amount of Bub1 was loaded in lanes 1 to 3, as indicated by Western analysis (Fig. 8B). However, autophosphorylation of Bub1 did not depend on the presence of Bub3 in the cell extract; Bub1 phosphorylation was still observed when it was immunoprecipitated from a *bub3* deletion mutant (Fig. 8A, lane 3). These data suggest that Bub3 has an activating effect on the Bub1 kinase but is not essential for its

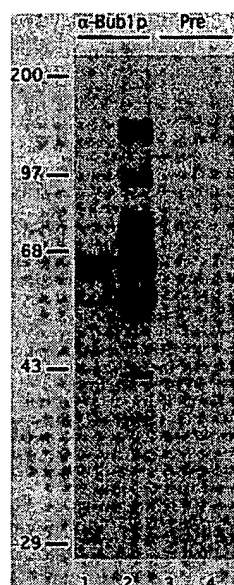


FIG. 6. Specificity of affinity-purified anti-Bub1 antiserum. Whole cell protein extracts from wild-type cells (MAY3317; lanes 1 and 3) or cells overexpressing *BUB1* and *BUB3* (MAY3188; lanes 2 and 4) were subjected to SDS-PAGE and transferred to nitrocellulose. Lanes 1 and 2 were probed with affinity-purified polyclonal antibodies raised to a portion of the *BUB1* gene product (see Materials and Methods). Lanes 3 and 4 were probed with affinity-purified preimmune serum from the same rabbit. Numbers at the left indicate approximate molecular masses in kilodaltons. The predicted molecular mass of full-length, unphosphorylated Bub1 is 118 kDa.

enzymatic activity. Note, however, that cells overproducing Bub1 and Bub3 together are inhibited for growth relative to those overproducing Bub1 alone. Cells devoid of Bub3 are also slowly growing but possibly for a different reason. Therefore,

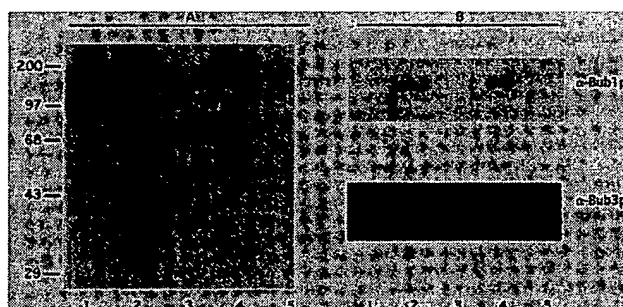


FIG. 7. Autophosphorylation assay. (A) Autoradiograph of immune complexes incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (see Materials and Methods). (B) Western blot of the same immune complexes, probed with an anti-Bub1 ($\alpha\text{-Bub1p}$) or anti-Bub3 ($\alpha\text{-Bub3p}$) antibody to assess recovery of these proteins. For both panels, immune complexes in lane 1 were formed with affinity-purified preimmune serum; all other immune complexes were from affinity-purified anti-Bub1 serum. Protein extracts were from wild-type strains with pGAL-*BUB1* pGAL-*BUB3* (MAY3188; lanes 1 and 2), pGAL-*bub1K733R* pGAL-*BUB3* (MAY3275; lane 4), or vector only (MAY3317; lane 5). Lane 3 is a fivefold dilution of lane 2. Approximate molecular mass standards for panel A are indicated (in kilodaltons) at the left. Bands in panel B are aligned with the corresponding positions in panel A.

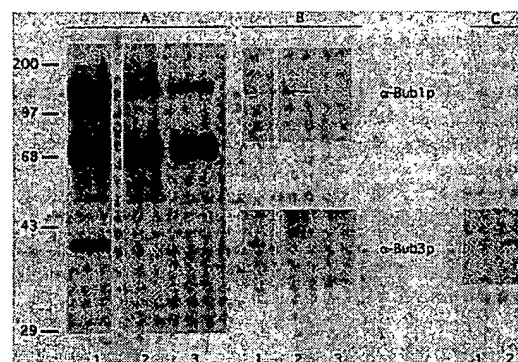


FIG. 8. Interaction of Bub3 with, and phosphorylation by, Bub1. (A) Autoradiograph of phosphorylated proteins from anti-Bub1 immune complexes; (B) Western analysis of the same immune complexes, to judge recovery of Bub1 ($\alpha\text{-Bub1p}$) and Bub3 ($\alpha\text{-Bub3p}$). In panels A and B, protein extracts were made from *BUB*⁺ pGAL-*BUB1* pGAL-*BUB3* (lane 1), *BUB*⁺ pGAL-*BUB1* (lane 2), and *bub3Δ* pGAL-*BUB1* (lane 3). (C) Coimmunoprecipitation of Bub3 with a fusion protein from the amino-terminal portion of Bub1. Both lanes represent immunoprecipitations with anti-Bub1 antibodies from *BUB*⁺ pGAL-*BUB3* cells (MAY3341). In lane 2, approximately 1 μg of a 64-kDa Bub1 partial fusion protein was added to the yeast protein extracts. Immune complexes were subjected to SDS-PAGE, blotted to nitrocellulose, and probed with the anti-Bub3 antibody.

the differences in Bub1 kinase activity observed in extracts from cells with various amounts of Bub3 may be an indirect consequence of physiological differences.

The region of Bub1 between amino acids 141 and 609 is sufficient for interaction with Bub3. It seemed possible that the amino-terminal portion of Bub1 contains a domain required for binding to Bub3. We tested the ability of a fusion protein

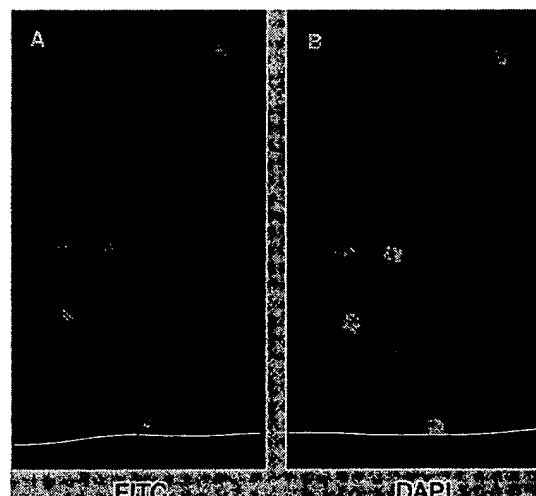


FIG. 9. Immunofluorescent staining of Bub1 in wild-type and *BUB*-overexpressing cells. Cells in panel A are labeled with affinity-pure anti-Bub1 antibody ($\alpha\text{-Bub1p}$) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody; cells in panel B are labeled with the fluorescent DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI). Both show the same field of pGAL-*BUB1* pGAL-*BUB3* cells (strain MAY3188) 12 h after induction with galactose (see Materials and Methods).

purified from *E. coli* including amino acids 141 through 609 of Bub1 to interact with Bub3. Note that this protein is the same as the protein used to generate the polyclonal anti-Bub1 antibodies used in this study. When immunoprecipitations were performed with these antibodies on extracts from cells overexpressing *BUB3* alone, Bub3 was not observed in the precipitate. However, when the fusion protein was added to the same protein extract before addition of antibodies, Bub3 was precipitated (Fig. 8C). These data indicate that a protein-binding domain sufficient for interaction with Bub3 is located between amino acids 141 and 609 of Bub1.

Overproduction of Bub1 results in its accumulation in the nucleus. The benomyl sensitivity caused by the overproduction of Bub1 and other evidence indicating a genetic interaction between Bub1, Bub3, and tubulin (9) (see Discussion) suggested that Bub1 might physically associate with microtubules. To determine the cellular localization of Bub1, in situ immunofluorescence was performed with affinity-pure antibodies against Bub1. This treatment revealed heavy nuclear staining in cells overexpressing *BUB1* and *BUB3*, but microtubule-specific staining was not detected (Fig. 9). This pattern was also observed in cells overexpressing *bub1K733R* and *BUB3* together and in *bub3Δ* and *BUB3* cells overexpressing *BUB1* alone (data not shown). No staining was detected in cells producing wild-type levels of Bub1.

DISCUSSION

The *BUB1* and *BUB3* genes were identified in a search for genes required for normal control of mitosis. Both proteins have been shown to be required for cell cycle arrest in response to spindle disruption (14). Several lines of evidence suggested that Bub1 and Bub3 act as part of a single pathway to maintain normal cell cycle timing. First, *BUB3* was originally cloned as an extra-copy suppressor of *bub1-1*. Second, the phenotypes of the *bub1Δ bub3Δ* double-deletion mutant were similar or equivalent to those of either single mutant. These mutants were viable but extremely slowly growing and sensitive to benomyl. Third, overexpression of the two genes together led to a sharp reduction in growth rate, although overproduction of the single gene products did not inhibit growth under permissive conditions. Fourth, these genes are each capable of allele-specific suppression of the *tub1-729* mutation when present in extra copy (9). Despite this genetic link, there is still no direct evidence for interaction of either Bub1 or Bub3 with tubulin. Finally, the two proteins coimmunoprecipitated, and the presence of Bub3 seemed to increase the in vitro activity of the Bub1 kinase.

The phenotypes of *bub1* and *mad3* mutants are similar (14, 20). Both exhibit premature cell cycle progression when the function of the mitotic spindle is compromised. The recently determined sequence of *MAD3* revealed that the predicted amino terminus of its product is closely related to the amino terminus of Bub1 (11). The first 360 amino acids of Bub1 are 35% identical to the corresponding region of Mad3, and this region includes a 147-amino-acid sequence that is 44% identical. This region overlaps with the region of Bub1 that we found is sufficient to interact with Bub3 in vitro (amino acids 141 through 609), suggesting the possibility that Mad3 also binds Bub3. Protein kinase consensus sequences were not found in the predicted *MAD3* gene product.

Bub1 and Bub3 activities are required to prevent premature exit from M phase when mitotic spindle function is compromised by microtubule depolymerization (14). It is possible that under these conditions, the Bub1 kinase is activated in some sort of signal transduction capacity. We have been unable to

detect any difference in Bub1 kinase activity between extracts obtained from nocodazole-treated and untreated cells (unpublished observations). However, it is possible that the overproduction of Bub1 necessary for its detection in this experiment overshadowed any normal in vivo regulatory effects. We can suggest two potential mechanisms of catalytic regulation of the Bub1 kinase. First, Bub1 is a member of a small subset of protein kinases with a tyrosine residue in the ATP binding site corresponding to position 716 in Bub1. Most of the other members of this group are closely related to p34^{cdc2}, the catalytic subunit of the MPF kinase (10). In some eukaryotes, MPF kinase activity is negatively regulated by phosphorylation of this tyrosine (3, 22–24). Although p34^{cdc2} is not a close relative of Bub1, it is possible that Bub1 is also regulated by phosphorylation of tyrosine 716. Second, it has been shown that catalytic regulation of some protein kinases is achieved through interaction of an autoinhibitory domain of the protein with the catalytic site (32). Kinase activity is stimulated by association with an allosteric activator. Bub3 may be such an activator of Bub1 or may direct the activation of Bub1 by an unidentified protein. Since the *BUB1* gene product is somewhat divergent from other known protein kinases, however, an alternative hypothesis is that Bub3 is a substrate-binding subunit within the Bub1-Bub3 kinase complex. In light of the fact that Bub1 produced in *bub3Δ* mutants is capable of autophosphorylation, this hypothesis seems highly unlikely. Bub3 may, however, direct association of Bub1 to its physiological substrate.

Elimination of MPF activity has been suggested to be required for exit from mitosis (8, 35). Since MPF activity has been shown to be improperly regulated in the *bub* mutants (14), the various MPF subunits may reasonably be viewed as candidate substrates and/or downstream effectors for Bub1 and Bub3. Many of the cyclin subunits of MPF are known to be phosphorylated in vivo. Although phosphorylation of *Xenopus* B-type cyclins has been shown to be nonessential for normal cell cycle function (15), a role for their phosphorylation in checkpoint control has not been ruled out.

In *S. pombe*, negative regulation of MPF activity is achieved in part through the phosphorylation of p34^{cdc2} on tyrosine 15 and threonine 14 (3, 22–24). Although the equivalent residues are phosphorylated with similar cell cycle kinetics on the *S. cerevisiae* homolog Cdc28 (2, 34), these phosphorylations are dispensable for normal arrest in G₁, S, or M phase. Indeed, mutations causing the change of the threonine and tyrosine to nonphosphorylatable residues has no influence on cell cycle timing or cell viability. In light of the deleterious nature of *bub1* or *bub3* mutations and their negative influence on MPF activity (14), it is unlikely that this Bub pathway influences cycle timing by affecting the phosphorylation state of these residues. In *S. pombe*, as well as other eukaryotes, the p40^{MO15} kinase is believed to catalyze phosphorylation of a third site on p34^{cdc2} (equivalent to *S. cerevisiae* position Thr-160), an event required for its activation (6, 26, 33). Since a p40^{MO15} functional homolog has not been identified in yeast isolates, Bub1 may be the primary *S. cerevisiae* Thr-160 kinase. Note, however, that sequence similarity between these kinases is limited. If not equivalent to Bub1, the yeast functional homolog of p40^{MO15} may be a candidate target for regulation by the Bub pathway. Finally, since it is clear that Bub3 is phosphorylated by Bub1 in vitro, Bub3 itself may be the only relevant substrate of Bub1.

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SEELEY
DECLARATION
EXHIBIT NO. 23



US006489137B2

(12) **United States Patent**
Seeley(10) **Patent No.:** **US 6,489,137 B2**
(45) **Date of Patent:** **Dec. 3, 2002**(54) **DETECTION OF LOSS OF THE WILD-TYPE HUBUB1 GENE**(75) **Inventor:** **Todd W. Seeley, Moraga, CA (US)**(73) **Assignee:** **Chiron Corporation, Emeryville, CA (US)**(*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.(21) **Appl. No.:** **09/095,881**(22) **Filed:** **Jun. 11, 1998**(65) **Prior Publication Data**

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(51) **Int. Cl.⁷** **C07H 21/04; A01N 63/00; C12N 15/00; C12N 15/63; C12P 21/06**(52) **U.S. Cl.** **435/69.1; 435/320.1; 435/455; 435/325; 536/23.1; 536/23.5**(58) **Field of Search** **536/23.1, 23.5; 435/320.1, 325, 69.1, 455, 6; 424/93.2**(56) **References Cited****U.S. PATENT DOCUMENTS**5,283,173 A 2/1994 Fields et al. 435/6
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* cited by examiner

Primary Examiner—Scott D. Priebe**Assistant Examiner**—Shin-Lin Chen(74) **Attorney, Agent, or Firm**—Jane E. R. Potter; Kimberlin L. Morley; Robert P. Blackburn(57) **ABSTRACT**

Methods are provided for assessing mutations and/or loss of the huBUB1 gene in human tumors. Loss of wild-type huBUB1 genes is involved in neoplastic development. Therapeutic regimens can be planned on the basis of the mutational status of huBUB1.

9 Claims, 2 Drawing Sheets**Serial No. 10/084,700****Filed 02/27/2002****SEELEY DECLARATION**
EXHIBIT NO. 23

scBUB1-kinase	DLYCIRGELGEGGYATVYLAESS-----QGHLRALKVEK---PASVWEYYIM
muBUB1-kinase	LVY-VNHLLGEGAFQAQVFEATHGDVRNAKSEQKCILKVQR---PANSWEFYIG
huBUB1-kinase	LVY-VHHLLGEGAFQAQVYEATQGDLDNAKNKQKFVLKVQK---PANPWEFYIG
pkc-kinase	TDFNFLMVLGKGSFGKVMLADRK-----GTEELYAIKILKKDVVIQD--DDVE
rs6k-kinase	ECFELLRVLGKGGYGKVFQVRKVT--GANTGKIFAMKVLKKAMIVRN--AKDT
cAPK	DQFERIKTLGTGSFGRVMLVKHKE-----TGNHYAMKILDKQKVVKL--KQIE
GSK-3-kinase	VSYTDTKVIGNGSFGVVYQAKLCD-----SGELVAIKKVLQDKRFKNR----E
	. * * . * *

Fig. 1

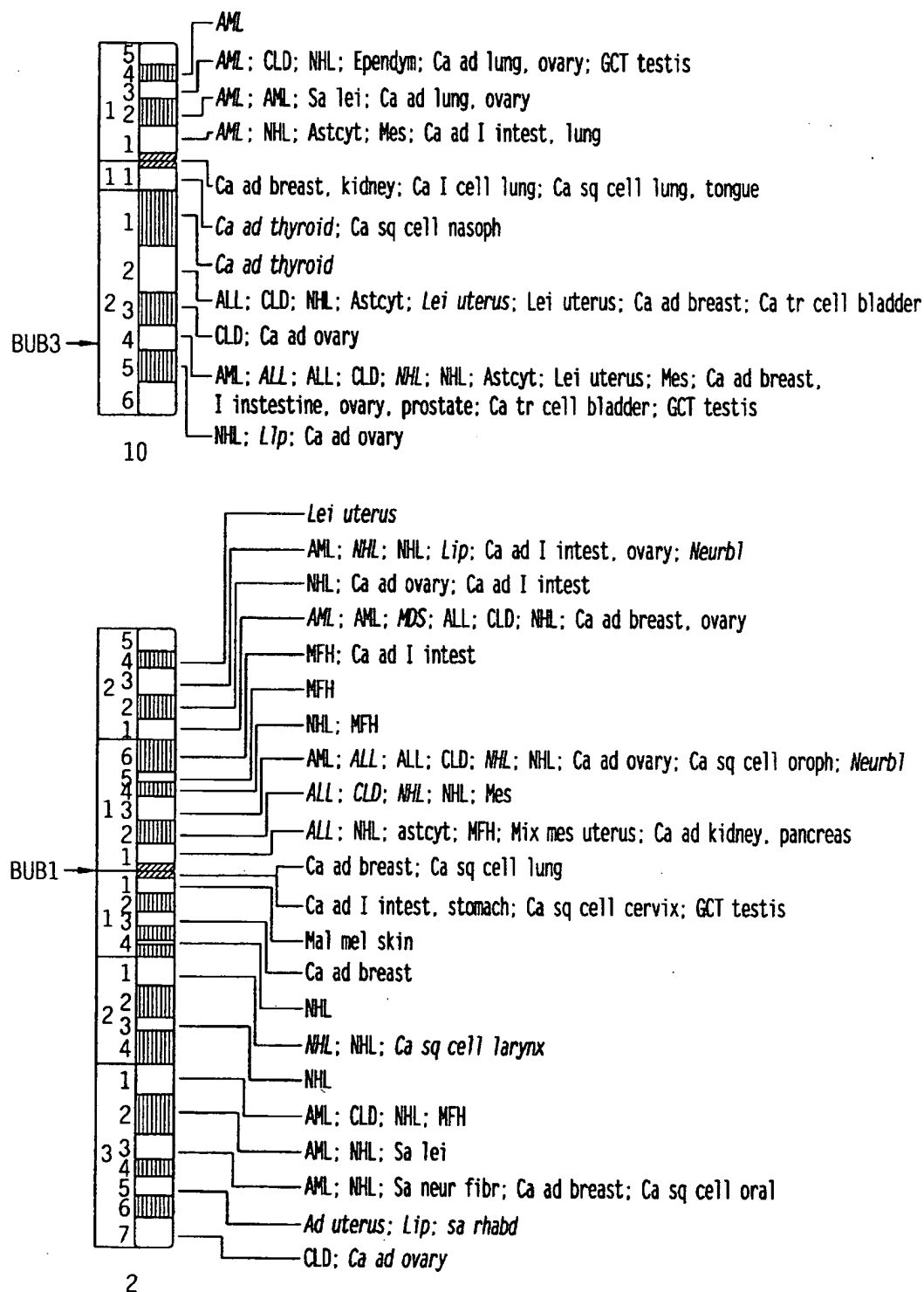


Fig. 2

1

DETECTION OF LOSS OF THE WILD-TYPE HUBUB1 GENE

This application claims the benefit of the following
copending provisional applications: Serial No. 60/049,068,
filed Jun. 11, 1997; Serial No. 60/068,102, filed Dec. 19,
1997; and Serial No. 60/070,182, filed Dec. 30, 1997. Each
of these applications is incorporated by reference herein.

TECHNICAL AREA OF THE INVENTION

The invention relates to the area of cancer diagnostics.
More particularly, the invention relates to detection of the
loss and/or alteration of wild-type huBUB1 genes in tumor
tissues.

BACKGROUND OF THE INVENTION

Genes and proteins involved in cell cycle regulation and
apoptosis have been found to be important in the develop-
ment of cancers. There is a continuing need in the art for
identification of components of cells which control the cell
cycle and apoptosis.

SUMMARY OF THE INVENTION

The object of this invention is to provide tools and
methods for diagnosing, prognosing, and treating neoplasia.
This and other objects of the invention are provided by one
or more of the embodiments described below.

One embodiment of the invention provides an isolated
and purified huBUB1 protein comprising an amino acid
sequence which is at least 85% identical to the amino acid
sequence shown in SEQ ID NO:2.

Another embodiment of the invention provides an isolated
and purified huBUB1 polypeptide which comprises at least
6 contiguous amino acids selected from an amino acid
sequence which is at least 85% identical to the amino acid
sequence shown in SEQ ID NO:2.

Yet another embodiment of the invention provides a
fusion protein comprising a first protein segment and a
second protein segment fused together with a peptide bond.
The first protein segment comprises at least 6 contiguous
amino acids of a huBUB1 protein having an amino acid
sequence which is at least 85% identical to the amino acid
sequence shown in SEQ ID NO:2.

Still another embodiment of the invention provides a
preparation of antibodies which specifically binds to a
huBUB1 protein.

Even another embodiment of the invention provides an
isolated and purified subgenomic polynucleotide comprising
at least 10 contiguous nucleotides selected from a nucleotide
sequence which is at least 85% identical to the nucleotide
sequence shown in SEQ ID NO:1.

A further embodiment of the invention provides a DNA
expression construct comprising an isolated and purified
subgenomic polynucleotide. The isolated and purified
subgenomic polynucleotide comprises at least 10 contiguous
nucleotides selected from a nucleotide sequence which is at
least 85% identical to the nucleotide sequence shown in
SEQ ID NO:1.

Another embodiment of the invention provides a host cell.
The host cell comprises an isolated and purified subgenomic
polynucleotide. The isolated and purified subgenomic poly-
nucleotide comprises at least 10 contiguous nucleotides
selected from a nucleotide sequence which is at least 85%
identical to the nucleotide sequence shown in SEQ ID NO:
1.

2

Still another embodiment of the invention provides a
method of diagnosing a neoplastic tissue of a human. A
tissue suspected of being neoplastic is isolated from a
human. Loss of a wild-type huBUB1 gene or an expression
product of a wild-type huBUB1 gene from the tissue is
detected. The loss of the wild-type huBUB1 gene or its
expression product indicates neoplasia of the tissue.

Yet another embodiment of the invention provides a
method of supplying wild-type huBUB1 gene function to a
cell which has lost said gene function by virtue of mutation
in a huBUB1 gene. All or a portion of a wild-type huBUB1
gene is introduced into a cell which has lost said gene
function. The portion of the wild-type huBUB1 gene is
required for non-neoplastic growth of the cell. The all or a
portion of the wild-type huBUB1 gene is expressed in the
cell.

Even another embodiment of the invention provides a pair
of single-stranded DNA primers. The pair of single-stranded
DNA primers allows synthesis of all or part of a huBUB1
gene coding sequence.

Another embodiment of the invention provides a nucleic
acid probe complementary to a wild-type huBUB1 gene as
shown in SEQ ID NO:1.

Yet another embodiment of the invention provides a
nucleic acid probe complementary to a mutant huBUB1
gene.

Still another embodiment of the invention provides a
method of detecting the presence of a neoplastic tissue in a
human. A body sample is isolated from a human. A mutant
huBUB1 gene or expression product is detected in the body
sample. Detection of a mutant huBUB1 gene or expression
product indicates the presence of a neoplastic tissue in the
human.

Another embodiment of the invention provides a method
of detecting genetic predisposition to cancer in a human. A
human sample selected from the group consisting of blood
and fetal tissue is isolated. DNA is extracted from the
sample. Loss of a wild-type huBUB1 gene from the DNA is
detected. Detection of the loss of a wild-type huBUB1 gene
indicates a genetic predisposition to cancer in the human.

A further embodiment of the invention provides a method
for identifying test compounds which interfere with
huBUB3-huBUB1 binding, said compounds being candi-
date therapeutic agents. A first protein, a second protein, and
a test compound are contacted. The first protein comprises at
least a portion of huBUB3 which binds to huBUB1 and the
second protein comprises at least a portion of huBUB1
which binds to huBUB3 or the first protein comprises at least
a portion of huBUB1 which binds to huBUB3 and the
second protein comprises at least a portion of huBUB3
which binds to huBUB1. The quantity of the first protein
which is bound to, is displaced from, or is prevented from
binding to, the second protein or the quantity of the second
protein which is bound to, displaced from, or is prevented
from binding to the first protein is determined. A compound
which diminishes the quantity of the first protein bound to
the second protein or the second protein bound to the first
protein, or which displaces the first protein bound to the
second protein or the second protein bound to the first
protein, or which prevents the first and second proteins from
binding, is identified as a candidate therapeutic agent.

Another embodiment of the invention provides a method
of identifying compounds which interfere with huBUB3-
huBUB1 binding. A cell is contacted with a compound to be
tested for its capacity to inhibit huBUB1-huBUB3 binding.
The cell comprises a first fusion protein comprising a

sequence-specific DNA-binding domain, a second fusion protein comprising a transcriptional activation domain, and a DNA construct comprising a reporter gene downstream from a DNA element which is recognized by the sequence-specific DNA binding-domain. The first fusion protein further comprises at least a portion of a huBUB3 protein which binds to a huBUB1 protein and the second fusion protein further comprises at least a portion of a huBUB1 protein which binds to a huBUB3 protein, or the first fusion protein further comprises at least a portion of a huBUB1 protein which binds to a huBUB3 protein and the second fusion protein further comprises at least a portion of a huBUB3 protein which binds to a huBUB1 protein. The amount of expression of the reporter gene in the presence of the compound is determined. A compound which decreases the amount of expression of the reporter gene is identified as a candidate therapeutic agent.

Still another embodiment of the invention provides a method of identifying test compounds which decrease the kinase activity of huBUB1. A huBUB1 protein is contacted with a test compound. The kinase activity of the huBUB1 protein is determined. A compound which decreases kinase activity of the huBUB1 protein is identified as a candidate therapeutic agent.

Even another embodiment of the invention provides a method of increasing the sensitivity of a tumor to an anti-tumor agent. The tumor is contacted with a compound which inhibits huBUB1 kinase activity or which inhibits huBUB1-huBUB3 binding. The sensitivity of the tumor to an anti-tumor agent is increased.

Another embodiment of the invention provides a method of expressing a huBUB1 subgenomic polynucleotide in a cell. The huBUB1 subgenomic polynucleotide is delivered to the cell. The huBUB1 subgenomic polynucleotide is expressed.

The present invention thus provides the art with the sequence of the human huBUB1 gene and protein. This information allows highly specific assays to be done to assess the neoplastic status of a particular tumor tissue.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1. FIG. 1 shows selected kinases, (SEQ ID NOS:9-15) including huBUB1 (SEQ ID NO:11), aligned using Clustal W.

FIG. 2. FIG. 2 shows the chromosomal locations of the huBUB1 and huBUB3 genes.

DETAILED DESCRIPTION

It is a discovery of the present invention that the human huBUB1 gene is involved in cell cycle control and apoptosis. A nucleotide sequence which encodes huBUB1 protein is shown in SEQ ID NO:1. huBUB1 protein binds to huBUB3 protein. The nucleotide and amino acid sequences of the huBUB3 gene and protein are shown in SEQ ID NOS:3 and 4, respectively. huBUB1 and the huBUB1-huBUB3 complex have kinase activity, and huBUB1 can autophosphorylate.

huBUB1 is mutated in cancer cells. Thus, loss of wild-type huBUB1 genes or function can be used to diagnose neoplasia. Furthermore, therapeutic regimens can be planned on the basis of the mutational status of huBUB1. Wild-type huBUB1 confers resistance to microtubule poisons such as vincristine, vinblastine, taxol, and taxotere. Thus, the finding of a mutation in huBUB1 will indicate that these agents can be used efficaciously. In contrast, finding a wild-type huBUB1 will suggest the use of other agents.

A huBUB1 protein has the amino acid sequence shown in SEQ ID NO:2. Any naturally occurring biologically active variants of this sequence which occur in human tissues are within the scope of this invention. Naturally occurring biologically active variants of full-length huBUB1 bind to huBUB3 and have kinase activity, including the ability to autophosphorylate. The huBUB3-binding domain of huBUB1 is located within amino acids 1-400 of SEQ ID NO:2, more particularly within amino acids 200-400 of SEQ ID NO:2. huBUB1 polypeptides differ in length from full-length huBUB1 and contain 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 45, 50, 75, 80, 90, or 100 or more contiguous amino acids of a huBUB1 protein.

Variants of huBUB1 protein and huBUB1 polypeptides can also occur. huBUB1 variants can be naturally or non-naturally occurring. Naturally occurring huBUB1 variants are found in humans or other species and comprise amino acid sequences which are substantially identical to the amino acid sequence shown in SEQ ID NO:2. Non-naturally occurring huBUB1 variants which retain substantially the same biological activities as naturally occurring huBUB1 variants are also included here. Preferably, naturally or non-naturally occurring huBUB1 variants have amino acid sequences which are at least 85%, 90%, or 95% identical to amino acid sequences shown in SEQ ID NO:2 and have similar biological properties, such as kinase activity, including the ability to autophosphorylate, and the ability to bind to huBUB3. More preferably, the molecules are at least 98% or 99% identical. Percent sequence identity between a wild-type huBUB1 protein or polypeptide and a huBUB1 variant is calculated by counting the number of amino acid matches between the wild-type and the variant and dividing the total number of matches by the total number of amino acid residues of the wild-type huBUB1 sequence.

Preferably, amino acid changes in huBUB1 variants are conservative amino acid changes, i.e., substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids.

It is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the biological properties of the resulting huBUB1 variant. Properties and functions of huBUB1 variants are of the same type as a huBUB1 protein or polypeptide comprising amino acid sequences of SEQ ID NO:2, although the properties and functions of variants can differ in degree. Whether an amino acid change results in a functional huBUB1 variant can readily be determined. For example, binding of a huBUB1 variant to huBUB3 can be detected using specific antibodies, which are disclosed herein. Kinase activity of a huBUB1 variant itself or in a complex with huBUB3 can also be measured, as described below.

huBUB1 variants include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties. huBUB1 variants also include allelic variants, species variants, and muteins. Trun-

cations or deletions of regions which do not affect the binding of huBUB1 to huBUB3 or the kinase activity of huBUB1 are also huBUB1 variants. Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art.

huBUB1 can be extracted, using standard biochemical methods, from huBUB1-producing human cells, such as spleen, thymus, prostate, testis, small intestine, colon, peripheral blood lymphocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney, or pancreas. An isolated and purified huBUB1 protein or polypeptide is separated from other compounds which normally associate with a huBUB1 protein or polypeptide in a cell, such as certain proteins, carbohydrates, lipids, or subcellular organelles. A preparation of isolated and purified huBUB1 proteins or polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure.

huBUB1 proteins and polypeptides can also be produced by recombinant DNA methods or by synthetic chemical methods. For production of recombinant huBUB1 proteins or polypeptides, coding sequences selected from the huBUB1 nucleotide sequence shown in SEQ ID NO:1, or variants of that sequence which encode huBUB1 protein, can be expressed in known prokaryotic or eukaryotic expression systems (see below). Bacterial, yeast, insect, or mammalian expression systems can be used, as is known in the art.

Alternatively, synthetic chemical methods, such as solid phase peptide synthesis, can be used to synthesize a huBUB1 protein or polypeptide. General means for the production of peptides, analogs or derivatives are outlined in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins—A Survey of Recent Developments*, Weinstein, B. ed., Marcell Dekker, Inc., publ., New York (1983). Moreover, substitution of D-amino acids for the normal L-stereoisomer can be carried out to increase the half-life of the molecule. huBUB1 variants can be similarly produced.

Non-naturally occurring fusion proteins comprising at least 6, 8, 10, 12, 15, 18, 20, 25, 50, 60, 75, 80, 90, or 100 or more contiguous huBUB1 amino acids can also be constructed. huBUB1 fusion proteins are useful for generating antibodies against huBUB1 amino acid sequences and for use in various assay systems. For example, huBUB1 fusion proteins can be used to identify proteins which interact with huBUB1 protein and influence its kinase activity or which interfere with the binding of huBUB1 to huBUB3. Physical methods, such as protein affinity chromatography, or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can also be used for this purpose. Such methods are well known in the art and can also be used as drug screens.

A huBUB1 fusion protein comprises two protein segments fused together by means of a peptide bond. The first protein segment comprises at least 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 75, 80, 90, or 100 or more contiguous amino acids of a huBUB1 protein. For example, a huBUB1 fusion protein can comprise the huBUB3 binding site and/or the kinase domain of huBUB1. These domains can be recognized by aligning the amino acid sequence of huBUB1 with that of the yeast homolog, ScBUB1. The amino acids can be selected from the amino acid sequence shown in SEQ ID NO:2 or from a biologically active variant of that sequence, such as those described above. The first protein segment can also comprise full-length huBUB1.

The second protein segment can be a full-length protein or a protein fragment or polypeptide. The fusion protein can be

labeled with a detectable marker, as is known in the art, such as a radioactive, fluorescent, chemiluminescent, or biotinylated marker. The second protein segment can be an enzyme which will generate a detectable product, such as β -galactosidase. The first protein segment can be N-terminal or C-terminal, as is convenient.

Techniques for making fusion proteins, either recombinantly or by covalently linking two protein segments, are also well known. Recombinant DNA methods can be used to prepare huBUB1 fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO:1 in proper reading frame with nucleotides encoding the second protein segment and expressing the DNA construct in a host cell, as described below.

Isolated and purified huBUB1 proteins, polypeptides, variants, or fusion proteins can be used as immunogens, to obtain preparations of antibodies which specifically bind to huBUB1 protein. The antibodies can be used, inter alia, to detect wild-type huBUB1 protein or huBUB1-huBUB3 complexes in human tissue and fractions thereof. The antibodies can also be used to detect the presence of mutations in the huBUB1 gene which result in under- or over-expression of a huBUB1 protein or in expression of a huBUB1 protein with altered size or electrophoretic mobility. Antibodies which specifically bind to huBUB3 protein can be similarly used and prepared, as described below for huBUB1 antibodies.

Preparations of polyclonal or monoclonal antibodies can be made using standard methods. Single-chain antibodies can also be prepared. Single-chain antibodies which specifically bind to huBUB1 proteins, polypeptides, variants, or fusion proteins can be isolated, for example, from single-chain immunoglobulin display libraries, as is known in the art. The library is "panned" against huBUB1 protein amino acid sequences, and a number of single chain antibodies which bind with high-affinity to different epitopes of huBUB1 protein can be isolated. Hayashi et al., 1995, *Gene* 160:129-30. Single-chain antibodies can also be constructed using a DNA amplification method, such as the polymerase chain reaction (PCR), using hybridoma cDNA as a template. Thirion et al., 1996, *Eur. J. Cancer Prev.* 5:507-11.

Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught in Coloma and Morrison, 1997, *Nat. Biotechnol.* 15:159-63. Construction of bivalent, bispecific single-chain antibodies is taught in Mallender and Voss, 1994, *J. Biol. Chem.* 269:199-206.

A nucleotide sequence encoding the single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into DNA expression constructs using standard recombinant DNA methods, and introduced into cells which express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology. Verhaar et al., 1995, *Int. J. Cancer* 61:497-501; Nicholls et al., 1993, *J. Immunol. Meth.* 165:81-91.

huBUB1-specific antibodies specifically bind to epitopes present in a full-length huBUB1 protein having the amino acid sequence shown in SEQ ID NO:2, to huBUB1 polypeptides, or to huBUB1 variants, either alone or as part of a fusion protein. Preferably, huBUB1 epitopes are not present in other human proteins. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, e.g., at least 15, 25, or 50 amino acids.

Antibodies which specifically bind to huBUB1 proteins, polypeptides, fusion proteins, or variants provide a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in Western blots or other immunochemical assays. Preferably, antibodies which specifically bind to huBUB1 epitopes do not detect other proteins in immunochemical assays and can immunoprecipitate a huBUB1 protein, polypeptide, fusion protein, or variant from solution.

Antibodies can be purified by methods well known in the art. Preferably, the antibodies are affinity purified, by passing the antibodies over a column to which a huBUB1 protein, polypeptide, variant, or fusion protein is bound. The bound antibodies can then be eluted from the column, for example, using a buffer with a high salt concentration.

Subgenomic polynucleotides contain less than a whole chromosome. Preferably, the polynucleotides are intron-free. Purified and isolated huBUB1 subgenomic polynucleotides can comprise at least 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, or 200 or more contiguous nucleotides selected from the nucleotide sequence shown in SEQ ID NO:1 or its complement. SEQ ID NO:1 is the coding sequence of a human huBUB1 gene. In one embodiment, a huBUB1 subgenomic polynucleotide comprises nucleotides which encode the kinase domain of huBUB1 or the huBUB3-binding site, as determined by aligning huBUB1 and ScBUB1 amino acid or nucleotide sequences.

The complement of the nucleotide sequence shown in SEQ ID NO:1 is a contiguous nucleotide sequence which forms Watson-Crick base pairs with the contiguous nucleotide sequence shown in SEQ ID NO:1. The complement of the nucleotide sequence shown in SEQ ID NO:1 (the antisense strand) is also a subgenomic polynucleotide, and can be used to provide huBUB1 antisense oligonucleotides. huBUB1 subgenomic polynucleotides also include polynucleotides which encode huBUB1-specific single-chain antibodies and ribozymes, or fusion proteins comprising huBUB1 amino acid sequences.

Degenerate nucleotide sequences encoding amino acid sequences of huBUB1 protein and or variants, as well as homologous nucleotide sequences which are at least 85%, 90%, 95%, 98%, or 99% identical to the nucleotide sequence shown in SEQ ID NO:1, are also huBUB1 subgenomic polynucleotides. Percent sequence identity between the sequence of a wild-type huBUB1 subgenomic polynucleotide and a homologous huBUB1 nucleotide sequence is calculated by counting the number of nucleotide matches between the wild-type and the homolog and dividing the total number of matches by the total number of nucleotides of the wild-type huBUB1 sequence. Typically, homologous huBUB1 sequences can be confirmed by hybridization under stringent conditions, as is known in the art.

huBUB1 subgenomic polynucleotides can be isolated and purified free from other nucleotide sequences using standard nucleic acid purification techniques. For example, restriction enzymes and probes can be used to isolate polynucleotide fragments which comprise nucleotide sequences encoding a huBUB1 protein. Isolated and purified subgenomic polynucleotides are in preparations which are free or at least 90% free of other molecules.

Complementary DNA molecules which encode huBUB1 proteins can be made using reverse transcriptase, with huBUB1 mRNA as a template. The polymerase chain reaction (PCR) or other amplification techniques can be used to obtain huBUB1 subgenomic polynucleotides, using either

human genomic DNA or cDNA as a template, as is known in the art. Alternatively, synthetic chemistry techniques can be used to synthesize huBUB1 subgenomic polynucleotides which comprise coding sequences for regions of huBUB1 proteins, single-chain antibodies, or ribozymes, or which comprise antisense oligonucleotides. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a huBUB1 protein comprising amino acid sequences of SEQ ID NO:2.

Purified and isolated huBUB1 subgenomic polynucleotides can be used as primers to obtain additional copies of the polynucleotides or as probes for identifying wild-type and mutant huBUB1 coding sequences. huBUB1 subgenomic polynucleotides can be used to express huBUB1 mRNA, protein, polypeptides, or fusion proteins and to generate huBUB1 antisense oligonucleotides and ribozymes.

A huBUB1 subgenomic polynucleotide comprising huBUB1 coding sequences can be used in an expression construct. Preferably, the huBUB1 subgenomic polynucleotide is inserted into an expression plasmid (for example, the Ecdyson system, pIND, In Vitro Gene). huBUB1 subgenomic polynucleotides can be propagated in vectors and cell lines using techniques well known in the art. huBUB1 subgenomic polynucleotides can be on linear or circular molecules. They can be on autonomously replicating molecules or on molecules without replication sequences. They can be regulated by their own or by other regulatory sequences, as are known in the art.

A host cell comprising a huBUB1 expression construct can then be used to express all or a portion of a huBUB1 protein. Host cells comprising huBUB1 expression constructs can be prokaryotic or eukaryotic. A variety of host cells are available for use in bacterial, yeast, insect, and human expression systems and can be used to express or to propagate huBUB1 expression constructs (see below). Expression constructs can be introduced into host cells using any technique known in the art. These techniques include transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, and calcium phosphate-mediated transfection.

A huBUB1 expression construct comprises a promoter which is functional in a chosen host cell. The skilled artisan can readily select an appropriate promoter from the large number of cell type-specific promoters known and used in the art. The expression construct can also contain a transcription terminator which is functional in the host cell. The expression construct comprises a polynucleotide segment which encodes all or a portion of the huBUB1 protein, variant, fusion protein, antibody, or ribozyme. The polynucleotide segment is located downstream from the promoter. Transcription of the polynucleotide segment initiates at the promoter. The expression construct can be linear or circular and can contain sequences, if desired, for autonomous replication.

Bacterial systems for expressing huBUB1 expression constructs include those described in Chang et al., *Nature* (1978) 275: 615, Goeddel et al., *Nature* (1979) 281: 544, Goeddel et al., *Nucleic Acids Res.* (1980) 8: 4057, EP 36,776, U.S. Pat. No. 4,551,433, deBoer et al., *Proc. Natl. Acad. Sci. USA* (1983) 80: 21-25, and Siebenlist et al., *Cell* (1980) 20: 269.

Expression systems in yeast include those described in Hinnen et al., *Proc. Natl. Acad. Sci. USA* (1978) 75: 1929; Ito

et al., *J. Bacteriol.* (1983) 153: 163; Kurtz et al., *Mol. Cell Biol.* (1986) 6: 142; Kunze et al., *J. Basic Microbiol.* (1985) 25: 141; Gleeson et al., *J. Gen. Microbiol.* (1986) 132: 3459; Roggenkamp et al., *Mol. Gen. Genet.* (1986) 202:302; Das et al., *J. Bacteriol.* (1984) 158: 1165; De Louvencourt et al., *J. Bacteriol.* (1983) 154: 737; Van den Berg et al., *Bio/Technology* (1990)8: 135; Kunze et al., *J. Basic Microbiol.* (1985)25:141; Cregg et al., *Mol. Cell Biol.* (1985) 5: 3376; U.S. Pat. Nos. 4,837,148, 4,929,555; Beach and Nurse, *Nature* (1981) 300: 706; Davidow et al., *Curr. Genet.* (1985)10: 380; Gaillardin et al., *Curr. Genet.* (1985) 10: 49; Ballance et al., *Biochem. Biophys. Res. Commun.* (1983) 112: 284-289; Tilburn et al., *Gene* (1983)26: 205-221; Yelton et al., *Proc. Natl. Acad. Sci. USA* (1984) 81: 1470-1474; Kelly and Hynes, *EMBO J.* (1985) 4: 475479; EP 244,234, and WO 91/00357.

Expression of huBUB1 expression constructs in insects can be carried out as described in U.S. Pat. No. 4,745,051, Friesen et al. (1986) "The Regulation of Baculovirus Gene Expression" in: *The Molecular Biology of Baculoviruses* (W. Doerfler, ed.), EP 127,839, EP 155,476, and Vlak et al., *J. Gen. Virol.* (1988) 69: 765-776; Miller et al., *Ann. Rev. Microbiol.* (1988) 42: 177; Carbonell et al., *Gene* (1988) 73: 409; Maeda et al., *Nature* (1985) 315: 592-594; Lebacqz-Verheyden et al., *Mol. Cell Biol.* (1988) 8: 3129; Smith et al., *Proc. Natl. Acad. Sci. USA* (1985) 82: 8404; Miyajima et al., *Gene* (1987) 58: 273; and Martin et al., *DNA* (1988) 7:99. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts are described in Luckow et al., *Bio/Technology* (1988) 6: 47-55; Miller et al., in *Genetic Engineering* (Setlow, J. K. et al. eds.), Vol. 8 Plenum Publishing, 1986), pp. 277-279, and Maeda et al., *Nature*, (1985) 315: 592-594.

Mammalian expression of huBUB1 expression constructs can be achieved as described in Dijkema et al., *EMBO J.* (1985) 4: 761; Gorman et al., *Proc. Natl. Acad. Sci. USA* (1982b) 79: 6777; Boshart et al., *Cell* (1985) 41: 521 and U.S. Pat. No. 4,399,216. Other features of mammalian expression of huBUB1 expression constructs can be facilitated as described in Ham and Wallace, *Meth. Enz.* (1979) 58: 44; Barnes and Sato, *Anal. Biochem.* (1980) 102: 255; U.S. Pat. Nos. 4,767,704, 4,657,866, 4,927,762, 4,560,655, WO 90/103430, WO 87/00195, and U.S. Pat. No. RE 30,985.

Subgenomic polynucleotides of the invention can also be used in gene delivery vehicles, for the purpose of delivering a huBUB1 mRNA or oligonucleotide (either with the sequence of native huBUB1 mRNA or its complement), full-length huBUB1 protein, huBUB1 fusion protein, huBUB1 polypeptide, or huBUB1-specific ribozyme or single-chain antibody, into a cell preferably a eukaryotic cell. According to the present invention, a gene delivery vehicle can be, for example, naked plasmid DNA, a viral expression vector comprising a huBUB1 subgenomic polynucleotide, or a huBUB1 subgenomic polynucleotide in conjunction with a liposome or a condensing agent.

In one embodiment of the invention, the gene delivery vehicle comprises a promoter and a huBUB1 subgenomic polynucleotide. Preferred promoters are tissue-specific promoters and promoters which are activated by cellular proliferation, such as the thymidine kinase and thymidylate synthase promoters. Other preferred promoters include promoters which are activatable by infection with a virus, such as the α - and β -interferon promoters, and promoters which are activatable by a hormone, such as estrogen. Other promoters which can be used include the Moloney virus LTR, the CMV promoter, and the mouse albumin promoter.

A huBUB1 gene delivery vehicle can comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from viruses such as astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the huBUB1 gene delivery vehicle is a recombinant retroviral vector. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann et al., *Cell* 33:153, 1983, Cane and Mulligan, *Proc. Nat'l. Acad. Sci. USA* 81:6349, 1984, Miller et al., *Human Gene Therapy* 1:5-14, 1990, U.S. Pat. Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Pat. No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res.* 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram et al., *Cancer Res.* 53:83-88, 1993; Takamiya et al., *J. Neurosci. Res.* 33:493-503, 1992; Baba et al., *J. Neurosurg.* 79:729-735, 1993 (U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805).

Particularly preferred retroviruses are derived from retroviruses which include avian leukosis virus (ATCC Nos. VR-535 and VR-247), bovine leukemia virus (VR-1315), murine leukemia virus (MLV), mink-cell focus-inducing virus (Koch et al., *J. Vir.* 49:828, 1984; and Oliffet al., *J. Vir.* 48:542, 1983), murine sarcoma virus (ATCC Nos. VR-844, 45010 and 45016), reticuloendotheliosis virus (ATCC Nos. VR-994, VR-770 and 45011), Rous sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus, endogenous feline retrovirus (e.g., RD114), and mouse or rat gL30 sequences used as a retroviral vector. Particularly preferred strains of MLV from which recombinant retroviruses can be generated include 4070A and 1504A (Hartley and Rowe, *J. Vir.* 19:19, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi (Ru et al., *J. Vir.* 67:4722, 1993; and Yantchev *Neoplasma* 26:397, 1979), Gross (ATCC No. VR-590), Kirsten (Albino et al., *J. Exp. Med.* 164:1710, 1986), Harvey sarcoma virus (Manly et al., *J. Vir.* 62:3540, 1988; and Albino et al., *J. Exp. Med.* 164:1710, 1986) and Rauscher (ATCC No. VR-998), and Moloney MLV (ATCC No. VR-190). A particularly preferred non-mouse retrovirus is Rous sarcoma virus. Preferred Rous sarcoma viruses include Bratislava (Manly et al., *J. Vir.* 62:3540, 1988; and Albino et al., *J. Exp. Med.* 164:1710, 1986), Bryan high titer (e.g., ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard (ATCC No. VR-140), Carr-Zilber (Adgighitov et al., *Neoplasma* 27:159, 1980), Engelbreth-Holm (Laurent et al., *Biochem Biophys Acta* 908:241, 1987), Harris, Prague (e.g., ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (e.g. ATCC Nos. VR-724, VR-725, VR-354) viruses.

Any of the above retroviruses can be readily utilized in order to assemble or construct retroviral huBUB1 gene delivery vehicles given the disclosure provided herein and standard recombinant techniques (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989, and Kunkle, *PNAS* 82:488, 1985) known in the art. Portions of retroviral huBUB1 expression vectors can be derived from different retroviruses. For example, retrovector LTRs can be derived from a murine sarcoma virus, a tRNA binding site from a Rous sarcoma virus, a packaging signal from a murine leukemia virus, and an origin of second strand synthesis

from an avian leukosis virus. These recombinant retroviral vectors can be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see Ser. No. 07/800,921, filed Nov. 29, 1991, now abandoned). Recombinant retroviruses can be produced which direct the site-specific integration of the recombinant retroviral genome into specific regions of the host cell DNA. Such site-specific integration can be mediated by a chimeric integrase incorporated into the retroviral particle (see Ser. No. 08/445,466 filed May 22, 1995, now abandoned). It is preferable that the recombinant viral gene delivery vehicle is a replication-defective recombinant virus.

Packaging cell lines suitable for use with the above-described retroviral gene delivery vehicles can be readily prepared (see Ser. No. 08/240,030, filed May 9, 1994, now abandoned; see also WO 92/05266) and used to create producer cell lines (also termed vector cell lines or "VCLs") for production of recombinant viral particles. In particularly preferred embodiments of the present invention, packaging cell lines are made from human (e.g., HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviral gene delivery vehicles which are capable of surviving inactivation in human serum. The construction of recombinant retroviral gene delivery vehicles is described in detail in WO 91/02805. These recombinant retroviral gene delivery vehicles can be used to generate transduction competent retroviral particles by introducing them into appropriate packaging cell lines (see Ser. No. 07/800,921, now abandoned). Similarly, adenovirus gene delivery vehicles can also be readily prepared and utilized given the disclosure provided herein (see also Berkner, *Biotechniques* 6:616-627, 1988, and Rosenfeld et al., *Science* 252:431-434, 1991, WO 93/07283, WO 93/06223, and WO 93/07282).

A huBUB1 gene delivery vehicle can also be a recombinant adenoviral gene delivery vehicle. Such vehicles can be readily prepared and utilized given the disclosure provided herein (see Berkner, *Biotechniques* 6:616, 1988, and Rosenfeld et al., *Science* 252:431, 1991, WO 93/07283, WO 93/06223, and WO 93/07282). Adeno-associated viral huBUB1 gene delivery vehicles can also be constructed and used to deliver huBUB1 amino acids or nucleotides. The use of adeno-associated viral gene delivery vehicles in vitro is described in Chatterjee et al., *Science* 258: 1485-1488 (1992), Walsh et al., *Proc. Nat'l. Acad. Sci.* 89: 7257-7261 (1992), Walsh et al., *J. Clin. Invest.* 94: 1440-1448 (1994), Flotte et al., *J. Biol. Chem.* 268: 3781-3790 (1993), Ponnazhagan et al., *J. Exp. Med.* 179: 733-738 (1994), Miller et al., *Proc. Nat'l. Acad. Sci.* 91: 10183-10187 (1994), Einerhand et al., *Gene Ther.* 2: 336-343 (1995), Luo et al., *Exp. Hematol.* 23: 1261-1267 (1995), and Zhou et al., *Gene Therapy* 3: 223-229 (1996). In vivo use of these vehicles is described in Flotte et al., *Proc. Nat'l. Acad. Sci.* 90: 10613-10617 (1993), and Kaplitt et al., *Nature Genet.* 8:148-153 (1994).

In another embodiment of the invention, a huBUB1 gene delivery vehicle is derived from a togavirus. Preferred togaviruses include alphaviruses, in particular those described in U.S. Ser. No. 08/405,627, filed Mar. 15, 1995, now abandoned WO 95/07994. Alpha viruses, including Sindbis and ELVS viruses can be gene delivery vehicles for huBUB1 polynucleotides. Alpha viruses are described in WO 94/21792, WO 92/10578 and WO 95/07994. Several different alphavirus gene delivery vehicle systems can be constructed and used to deliver huBUB1 subgenomic polynucleotides to a cell according to the present invention.

Representative examples of such systems include those described in U.S. Pat. Nos. 5,091,309 and 5,217,879. Particularly preferred alphavirus gene delivery vehicles for use in the present invention include those which are described in WO 95/07994, and U.S. Ser. No. 08/405,627 now abandoned.

Preferably, the recombinant viral vehicle is a recombinant alphavirus viral vehicle based on a Sindbis virus. Sindbis constructs, as well as numerous similar constructs, can be readily prepared essentially as described in U.S. Ser. No. 08/198,450. Sindbis viral gene delivery vehicles typically comprise a 5' sequence capable of initiating Sindbis virus transcription, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region inactivated so as to prevent subgenomic fragment transcription, and a Sindbis RNA polymerase recognition sequence. Optionally, the viral junction region can be modified so that subgenomic polynucleotide transcription is reduced, increased, or maintained. As will be appreciated by those in the art, corresponding regions from other alphaviruses can be used in place of those described above.

The viral junction region of an alphavirus-derived gene delivery vehicle can comprise a first viral junction region which has been inactivated in order to prevent transcription of the subgenomic polynucleotide and a second viral junction region which has been modified such that subgenomic polynucleotide transcription is reduced. An alphavirus-derived vehicle can also include a 5' promoter capable of initiating synthesis of viral RNA from cDNA and a 3' sequence which controls transcription termination.

Other recombinant togaviral gene delivery vehicles which can be utilized in the present invention include those derived from Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleburg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described in U.S. Pat. Nos. 5,091,309 and 5,217,879 and in WO 92/10578. The Sindbis vehicles described above, as well as numerous similar constructs, can be readily prepared essentially as described in U.S. Ser. No. 08/198,450 now abandoned.

Other viral gene delivery vehicles suitable for use in the present invention include, for example, those derived from poliovirus (Evans et al., *Nature* 339:385, 1989, and Sabin et al., *J. Biol. Standardization* 1: 115, 1973) (ATCC VR-58); rhinovirus (Arnold et al., *J. Cell. Biochem.* L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus Fisher-Hoch et al., *PNAS* 86:317, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86, 1989; Flexner et al., *Vaccine* 8:17, 1990; U.S. Pat. Nos. 4,603,112 and 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan et al., *Nature* 277:108, 1979) (ATCC VR-305), (Madzak et al., *J. Gen. Vir.* 73:1533, 1992); influenza virus (Luytjes et al., *Cell* 59:1107, 1989; McMichael et al., *The New England Journal of Medicine* 309:13, 1983; and Yap et al., *Nature* 273:238, 1978) (ATCC VR-797); parvovirus such as adeno-associated virus (Samulski et al., *J. Vir.* 63:3822, 1989, and Mendelson et al., *Virology* 166:154, 1988) (ATCC VR-645); herpes simplex virus (Kit et al., *Adv. Exp. Med. Biol.* 215:219, 1989) (ATCC VR-977; ATCC VR-260); *Nature* 277: 108, 1979); human immunodeficiency virus (EPO 386,882, Buchschacher et al., *J. Vir.* 66:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); A (ATCC VR-67; ATCC VR-1247), Aura (ATCC VR-368), Bebaru virus (ATCC VR-600; ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64; ATCC VR-1241), Fort Morgan (ATCC VR-924),

Getah virus (ATCC VR-369; ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mucambo virus (ATCC VR-580; ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372; ATCC VR-1245), Tonate (ATCC VR-925), Trinité (ATCC VR-469), Una (ATCC VR-374), Whataroa (ATCC VR-926), Y-62-33 (ATCC VR-375), ONyong virus, Eastern encephalitis virus (ATCC VR-65; ATCC VR-1242), Western encephalitis virus (ATCC VR-70; ATCC VR-1251; ATCC VR-622; ATCC VR-1252), and coronavirus (Hamre et al., *Proc. Soc. Exp. Biol. Med.* 121:190, 1966) (ATCC VR-740).

A subgenomic huBUB1 polynucleotide of the invention can also be combined with a condensing agent to form a gene delivery vehicle. In a preferred embodiment, the condensing agent is a polycation, such as polylysine, polyarginine, polyornithine, protamine, spermine, spermidine, and putrescine. Many suitable methods for making such linkages are known in the art (see, for example, Ser. No. 08/366,787, filed Dec. 30, 1994 now abandoned).

In an alternative embodiment, a huBUB1 subgenomic polynucleotide is associated with a liposome to form a gene delivery vehicle. Liposomes are small, lipid vesicles comprised of an aqueous compartment enclosed by a lipid bilayer, typically spherical or slightly elongated structures several hundred Angstroms in diameter. Under appropriate conditions, a liposome can fuse with the plasma membrane of a cell or with the membrane of an endocytic vesicle within a cell which has internalized the liposome, thereby releasing its contents into the cytoplasm. Prior to interaction with the surface of a cell, however, the liposome membrane acts as a relatively impermeable barrier which sequesters and protects its contents, for example, from degradative enzymes. Additionally, because a liposome is a synthetic structure, specially designed liposomes can be produced which incorporate desirable features. See Stryer, *Biochemistry*, pp. 236-240, 1975 (W. H. Freeman, San Francisco, Calif.); Szoka et al., *Biochim. Biophys. Acta* 600:1, 1980; Bayer et al., *Biochim. Biophys. Acta* 550:464, 1979; Rivnay et al., *Meth. Enzymol.* 149:119, 1987; Wang et al., *PNAS* 84: 7851, 1987; Plant et al., *Anal. Biochem.* 176:420, 1989, and U.S. Pat. No. 4,762,915. Liposomes can encapsulate a variety of nucleic acid molecules including DNA, RNA, plasmids, and expression constructs comprising huBUB1 subgenomic polynucleotides such those disclosed in the present invention.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7416, 1987), mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA* 86:6077-6081, 1989), and purified transcription factors (Debs et al., *J. Biol. Chem.* 265:10189-10192, 1990), in functional form. Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy]propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, N.Y. See also Felgner et al., *Proc. Natl. Acad. Sci. USA* 91: 5148-5152.87, 1994. Other commercially available liposomes include Transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g. Szoka et al., *Proc. Natl. Acad. Sci. USA* 75:4194-4198, 1978; and WO 90/11092 for descriptions of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham,

Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., *Methods of Immunology* (1983), Vol. 101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* 87:3410-3414, 1990; Papahadjopoulos et al., *Biochim. Biophys. Acta* 394:483, 1975; Wilson et al., *Cell* 17:77, 1979; Deamer and Bangham, *Biochim. Biophys. Acta* 443:629, 1976; Ostro et al., *Biochem. Biophys. Res. Commun.* 76:836, 1977; Fraley et al., *Proc. Natl. Acad. Sci. USA* 76:3348, 1979; Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* 76:145, 1979; Fraley et al., *J. Biol. Chem.* 255:10431, 1980; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* 75:145, 1979; and Schaefer-Ridder et al., *Science* 215:166, 1982.

In addition, lipoproteins can be included with a huBUB1 subgenomic polynucleotide for delivery to a cell. Examples of such lipoproteins include chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Modifications of naturally occurring lipoproteins can also be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing lipoprotein receptors. Preferably, if lipoproteins are included with a polynucleotide, no other targeting ligand is included in the composition.

In another embodiment, naked huBUB1 subgenomic polynucleotide molecules are used as gene delivery vehicles, as described in WO 90/11092 and U.S. Pat. No. 5,580,859. Such gene delivery vehicles can be either huBUB1 DNA or RNA and, in certain embodiments, are linked to killed adenovirus. Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992. Other suitable vehicles include DNA-ligand (Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989), lipid-DNA combinations (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), liposomes (Wang et al., *Proc. Natl. Acad. Sci. USA* 84:7851-7855, 1987) and microprojectiles (Williams et al., *Proc. Natl. Acad. Sci. USA* 88:2726-2730, 1991).

One can increase the efficiency of naked huBUB1 subgenomic polynucleotide uptake into cells by coating the polynucleotides onto biodegradable latex beads. This approach takes advantage of the observation that latex beads, when incubated with cells in culture, are efficiently transported and concentrated in the perinuclear region of the cells. The beads will then be transported into cells when injected into muscle. huBUB1 subgenomic polynucleotide-coated latex beads will be efficiently transported into cells after endocytosis is initiated by the latex beads and thus increase gene transfer and expression efficiency. This method can be improved further by treating the beads to increase their hydrophobicity, thereby facilitating the disruption of the endosome and release of huBUB1 subgenomic polynucleotides into the cytoplasm.

huBUB1 or huBUB3 activity can be decreased in a cell by contacting the cell with a reagent which binds to an expression product of huBUB1 or huBUB3, respectively. In one embodiment of the invention, the reagent is a ribozyme, an

RNA molecule with catalytic activity. See, e.g., Cech, *Science* 236: 1532-1539; 1987; Cech, *Ann. Rev. Biochem.* 59:543-568; 1990, Cech, *Curr. Opin. Struct. Biol.* 2: 605-609; 1992, Couture and Stinchcomb, *Trends Genet.* 12: 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (e.g., Haseloff et al., U.S. Pat. No. 5,641,673).

The coding sequence of a huBUB1 or huBUB3 genes can be used to generate ribozymes which will specifically bind to mRNA transcribed from the huBUB1 or huBUB3 genes. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff, J. et al. *Nature* 334:585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach, et al., EP 321,201). The nucleotide sequences shown in SEQ ID NOS:1 and 3 provide a source of suitable hybridization region sequences. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related; thus, upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct, as is known in the art and described above. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce the ribozyme-containing DNA construct into cells in which it is desired to decrease huBUB1 or huBUB3 expression, as described above. Alternatively, if it is desired that the cells stably retain the DNA construct, it can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. The DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff et al., U.S. Pat. No. 5,641,673, ribozymes can be engineered so that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes can also be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

In another embodiment of the invention, the level of huBUB1 or huBUB3 gene expression is decreased using an antisense oligonucleotide sequence. The antisense sequence is complementary to at least a portion of the sequence encoding huBUB1 or huBUB3 selected from the nucleotide sequences shown in SEQ ID NOS:1 or 3. Preferably, the antisense oligonucleotide sequence is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences can also be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into cells as described above to decrease the level of huBUB1 or huBUB3 in the cells.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer,

by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such as alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. See Brown, *Meth. Mol. Biol.* 20:1-8, 1994; Sonveaux, *Meth. Mol. Biol.* 26:1-72, 1994; Uhlmann et al., *Chem. Rev.* 90:543-583, 1990.

Although precise complementarity is not required for successful duplex formation between an antisense molecule and the complementary coding sequence of a huBUB1 or huBUB3 gene, antisense molecules with no more than one mismatch are preferred. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular coding sequence.

Antisense oligonucleotides can be modified without affecting their ability to hybridize to a huBUB1 or huBUB3 coding sequence. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3',5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, can also be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. See, e.g., Agrawal et al., *Trends Biotechnol.* 10:152-158, 1992; Uhlmann et al., *Chem. Rev.* 90:543-584, 1990; Uhlmann et al., *Tetrahedron. Lett.* 215:3539-3542, 1987.

Antibodies of the invention which specifically bind to huBUB1, particularly single-chain antibodies, can also be used to alter levels of huBUB1. Antibodies similarly prepared against huBUB3 can be used to alter levels of huBUB3. The antibodies prevent huBUB1 and huBUB3 from binding. Polynucleotides encoding single-chain antibodies of the invention can be introduced into cells as described above.

Preferably, the mechanism used to decrease the level of huBUB1 or huBUB3 expression, whether ribozyme, antisense oligonucleotide sequence, or antibody, decreases the level of gene expression by at least 50%, 60%, 70%, or 80%. Most preferably, the level of gene expression is decreased by at least 90%, 95%, 99%, or 100%. The effectiveness of the mechanism chosen to decrease the level of gene expression can be assessed using methods well known in the art, such as hybridization of nucleotide probes to huBUB1 or huBUB3 mRNA, quantitative RT-PCR, or detection of huBUB1 or huBUB3 protein using specific antibodies of the invention.

Compositions comprising huBUB1 or huBUB3 antibodies, ribozymes, or antisense oligonucleotides can optionally comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized macromolecules, such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutically acceptable salts can also be used in huBUB1 or huBUB3 compositions, for example, mineral salts such as hydrochlorides, hydrobromides,

phosphates, or sulfates, as well as salts of organic acids such as acetates, propionates, malonates, or benzoates. huBUB1 or huBUB3 compositions can also contain liquids, such as water, saline, glycerol, and ethanol, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes, such as those described in U.S. Pat. No. 5,422,120, WO 95/13796, WO 91/14445, or EP 524,968 B1, can also be used as a carrier for a huBUB1 composition.

Typically, a huBUB1 or huBUB3 composition is prepared as an injectable, either as a liquid solution or suspension; however, solid forms suitable for solution or suspension in liquid vehicles prior to injection can also be prepared. A huBUB1 or huBUB3 composition can also be formulated into an enteric coated tablet or gel capsule according to known methods in the art, such as those described in U.S. Pat. No. 4,853,230, EP 225,189, AU 9,224,296, and AU 9,230,801.

Mutations in huBUB1 are diagnostic of neoplasia. According to a diagnostic method of the present invention, loss of the wild-type huBUB1 gene is detected. The loss may be due to either deletional and/or point mutational events. If only a single huBUB1 allele is mutated, an early neoplastic state may be indicated. However, if both alleles are mutated then a late neoplastic state may be indicated. Point mutational events may occur in regulatory regions, such as in the promoter of the huBUB1 gene, leading to loss or diminution of expression of the huBUB1 mRNA. This can be determined using assays for quantitating huBUB1 expression.

In order to detect the loss of the huBUB1 wild-type gene in a tissue, it is helpful to isolate the tissue free from surrounding normal tissues. Means for enriching a tissue preparation for tumor (or cancer) cells are known in the art. For example, the tissue may be isolated from paraffin or cryostat sections. Cancer cells may also be separated from normal cells by flow cytometry. These as well as other techniques for separating tumor from normal cells are well known in the art. If the tumor tissue is highly contaminated with normal cells, detection of mutations is more difficult.

Detection of point mutations may be accomplished by molecular cloning of the huBUB1 allele (or alleles) present in the tumor tissue and sequencing that allele(s) using techniques well known in the art. Alternatively, the polymerase chain reaction or other amplification techniques can be used to amplify huBUB1 gene sequences directly from a genomic DNA preparation from the tumor tissue. The DNA sequence of the amplified sequences can then be determined. The polymerase chain reaction itself is well known in the art. See, e.g., Saiki et al., *Science*, 239, 487, 1988; U.S. Pat. Nos. 4,683,203; and 4,683,195. Specific primers which can be used in order to amplify the huBUB1 gene will be discussed in more detail below.

Specific deletions of huBUB1 genes can also be detected. For example, restriction fragment length polymorphism (RFLP) probes for the huBUB1 gene or surrounding marker genes can be used to score loss of a huBUB1 allele. Other techniques for detecting deletions, as are known in the art can be used.

Loss of wild-type huBUB1 genes can also be detected on the basis of the loss of a wild-type expression product of the huBUB1 gene. Such expression products include both the mRNA as well as the huBUB1 protein product itself. Point mutations can be detected by sequencing the mRNA directly or via molecular cloning of cDNA made from the mRNA. The sequence of the cloned cDNA can be determined using DNA sequencing techniques which are well known in the art. The cDNA can also be sequenced via the polymerase chain reaction (PCR) which will be discussed in more detail below.

Alternatively, mismatch detection can be used to detect point mutations in the huBUB1 gene or its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of tumors. An example of a mismatch cleavage technique is the RNase protection method, which is described in detail in Winter et al., *Proc. Natl. Acad. Sci. U.S.A.* 82, 7575 (1985) and Meyers et al., *Science* 230, 1242 (1985). In the practice of the present invention the method involves the use of a labeled riboprobe which is complementary to the human wild-type huBUB1 gene. The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is separated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full-length duplex RNA for the riboprobe and the huBUB1 mRNA or DNA. The riboprobe need not be the full length of the huBUB1 mRNA or gene but can be a segment of either. If the riboprobe comprises only a segment of the huBUB1 mRNA or gene it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., *Proc. Natl. Acad. Sci. U.S.A.* 85, 4397 (1988) and Shenk et al., *Proc. Natl. Acad. Sci. U.S.A.* 72, 989 (1975). Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., Cariello, *Human Genetics* 42, 726 (1988). With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified before hybridization using PCR or other amplification techniques, as is known in the art.

DNA sequences of the huBUB1 gene from the tumor tissue which have been amplified can also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the huBUB1 gene sequence harboring a known mutation. For example, one oligomer can be at least about 15, 18, 20, 30, or 50 nucleotides in length, corresponding to a portion of the huBUB1 gene sequence. By use of a battery of such allele-specific probes, amplification products can be screened to identify the presence of a previously identified mutation in the huBUB1 gene. Hybridization of allele-specific probes with amplified huBUB1 sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

Loss of wild-type huBUB1 genes can also be detected by screening for loss of wild-type huBUB1 protein function. Although all of the functions which the huBUB1 protein undoubtedly possesses have yet to be elucidated, at least two specific functions are known. Protein huBUB1 binds to huBUB3. Loss of the ability of the huBUB1 protein to bind to huBUB3 indicates a mutational alteration in the protein which reflects a mutational alteration of the huBUB1 gene itself. Similarly, loss of kinase activity of huBUB1 can be monitored as a means of detecting mutations. Alternatively, a panel of monoclonal or single-chain antibodies could be used in which epitopes involved in huBUB1 functions are represented by a monoclonal or single-chain antibody. Loss or perturbation of binding of huBUB1 to a monoclonal antibody in the panel would indicate mutational alteration of

the huBUB1 protein and thus of the huBUB1 gene itself. Any means for detecting an altered huBUB1 protein can be used to detect loss of wild-type huBUB1 genes.

Mutant huBUB1 genes or gene products can also be detected in body samples, such as serum or stool, or other body fluids, such as urine and sputum. The same techniques discussed above for detection of mutant huBUB1 genes or gene products in tissues can be applied to other body samples. By screening such body samples, a simple early diagnosis can be achieved for many types of cancers. In addition, the progress of chemotherapy can be monitored more easily by testing such body samples for mutant huBUB1 genes or gene products.

The method of the present invention for diagnosis of neoplastic tissue is applicable across a broad range of tumors. These include lung, breast, brain, colorectal, bladder, mesenchyme, prostate, liver as well as stomach tumors. In addition, the method can be used in leukemias and osteosarcomas. It thus appears that the huBUB1 gene has a role in the development of a broad range of tumors. The methods of diagnosis of the present invention are applicable to any tumor in which huBUB1 has a role in tumorigenesis. The diagnostic method of the present invention is useful for clinicians so that they can decide upon an appropriate course of treatment. For example, a tumor displaying loss of wild-type huBUB1 alleles suggests the use of mitotic poison-type chemotherapy. Wild-type huBUB1 in a tumor suggests that other types of anti-cancer therapies should be used.

The invention also provides diagnostic kits. A kit of the present invention is useful for determination of the nucleotide sequence of a huBUB1 gene using the polymerase chain reaction or other amplification technique. A kit comprises one or a set of pairs of single-stranded DNA primers which can be annealed to sequences within or surrounding the huBUB1 gene in order to prime amplifying DNA synthesis of the huBUB1 gene itself. The complete set allows synthesis of all of the nucleotides of the huBUB1 gene coding sequences, although isolated primers for selected portions can also be used. The set of primers may or may not allow synthesis of both intron and exon sequences. However, it should allow synthesis of all exon sequences.

In order to facilitate subsequent cloning of amplified sequences, primers may have restriction enzyme sites appended to their 5' ends. Thus, all nucleotides of the primers are derived from huBUB1 sequences or sequences adjacent to huBUB1 except the few nucleotides necessary to form a restriction enzyme site. Such enzymes and sites are well known in the art. The primers themselves can be synthesized using techniques which are well known in the art. Generally, the primers can be made using synthesizing machines which are commercially available. In a preferred embodiment, the primer pairs comprise: TWS86 (5'ATCATTCATGGAGACATTAAGCC-3') (SEQ ID NO:5) and TWS87 (5'-TTTCATGTAAGAGCCAAAGAGCAT-3') (SEQ ID NO:6).

Nucleotide probes according to the present invention comprise at least about 10, 12, 14, 16, 18, 20, 25, or 30 contiguous nucleotides of huBUB1. They can also contain labeling moieties with which the probes can be detected, including but not limited to radiolabels, fluorescent labels, and enzymatic labels. Nucleotide probes provided by the present invention are useful in the RNase protection method, for detecting point mutations already discussed above. Probes can also be used to detect mismatches with a huBUB1 gene or mRNA using other techniques. Mis-

matches can be detected using other enzymes (e.g., S1 nuclease), chemicals (e.g., hydroxylamine or osmium tetroxide and piperidine), or changes in electrophoretic mobility of mismatched hybrids as compared to totally matched hybrids. These techniques are known in the art. See Cotton, supra; Shenk, supra; Myers, supra; Winter, supra; and Novack et al., *Proc. Natl. Acad. Sci. U.S.A.* 83, 586 (1986). If a riboprobe is used to detect mismatches with mRNA, it is complementary to the mRNA of the human wild-type huBUB1 gene. The riboprobe thus is an anti-sense probe in that it does not code for the huBUB1 protein because it is of the opposite polarity to the sense strand. The riboprobe generally will be radioactively labeled; such labeling can be accomplished by any means known in the art. If the riboprobe is used to detect mismatches with DNA it can be of either polarity, sense or anti-sense. Similarly, DNA probes also may be used to detect mismatches. Probes may also be complementary to mutant alleles of huBUB1. These probes are useful to detect similar mutations in other patients on the basis of hybridization rather than mismatches. These probes are discussed above and referred to as allele-specific probes.

Genetic predisposition to cancers or neoplasia can be ascertained by testing normal tissues of humans. For example, a person who has inherited a germline huBUB1 mutation would be prone to develop cancers. This predisposition can be determined by testing DNA from any tissue of the person's body. Most simply, blood can be drawn and DNA extracted from cells of the blood. Loss of a wild-type huBUB1 allele, either by point mutation, deletion, or insertion can be detected by any of the means discussed above. DNA can also be extracted and tested from fetal tissues for this purpose.

According to the present invention a method is also provided of supplying wild-type huBUB1 function to a cell which carries mutant huBUB1 alleles. The wild-type huBUB1 gene or a part of the gene can be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation the gene will be expressed by the cell from the extrachromosomal location. If a gene portion is introduced and expressed in a cell carrying a mutant huBUB1 allele, the gene portion should encode a part of the huBUB1 protein which is required for non-neoplastic growth of the cell. The portion of huBUB1 protein which is required for non-neoplastic growth can be readily determined, for example, by transfecting DNA expression constructs comprising portions of huBUB1 protein, such as the huBUB3 binding domain or the kinase domain, into neoplastic cell lines in vitro and observing alterations in cellular morphology or lowered rates of cell division, as is known in the art.

More preferred is the situation where the wild-type huBUB1 gene or a part of it is introduced into the mutant cell in such a way that it recombines with the endogenous mutant huBUB1 gene present in the cell. Such recombination would require a double recombination event which would result in the correction of the huBUB1 gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art and any suitable vector may be used.

A composition comprising all or a portion of a huBUB1 subgenomic polynucleotide or polypeptide or other molecule which has huBUB1 activity can be supplied to cells which carry mutant huBUB1 alleles. The active molecules can be introduced into the cells by local or systemic administration, including injection, oral administration, particle gun, or catheterized administration, and topical admin-

istration. Alternatively, some such active molecules can be taken up by the cells, actively or by diffusion.

Various methods can be used to administer a huBUB1 therapeutic composition directly to a specific site in the body. For treatment of a tumor, for example, an appropriate huBUB1 composition injected several times in several different locations within the body of the tumor. Alternatively, arteries which serve the tumor can be identified, and a huBUB1 composition can be injected into such an artery in order to deliver the composition to the tumor.

A tumor which has a necrotic center can be aspirated, and a huBUB1 composition can be injected directly into the now empty center of the tumor. A huBUB1 composition can also be administered directly to the surface of a tumor, for example, by topical application of the composition. X-ray imaging can be used to assist in certain of these delivery methods. Combination therapeutic agents, including a huBUB1 protein or polypeptide or a huBUB1 subgenomic polynucleotide, can be administered simultaneously or sequentially together with other therapeutic agents.

huBUB1 compositions can be delivered to specific tissues using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques are taught in, for example, Findeis et al. *Trends in Biotechnol.* 11, 202-05, (1993); Chiou et al., *Gene Therapeutics: Methods and Applications of Direct Gene Transfer* (J. A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* 263,621-24,1988; Wu et al., *J. Biol. Chem.* 269, 54246, 1994; Zenke et al., *Proc. Natl. Acad. Sci. U.S.A.* 87,3655-59,1990; Wu et al., *J. Biol. Chem.* 266, 338-42,1991.

Both the dose of a particular huBUB1 composition and the means of administering the composition can be determined based on specific qualities of the huBUB1 composition, the condition, age, and weight of the patient, the progression of the particular disease being treated, and other relevant factors. If the composition contains huBUB1 proteins, polypeptides, or antibodies, effective dosages of the composition are in the range of about 5 μ g to about 50 μ g/kg of patient body weight, about 50 μ g to about 5 mg/kg, about 100 μ g to about 500 μ g/kg of patient body weight, and about 200 to about 250 μ g/kg.

Compositions containing huBUB1 subgenomic polynucleotides, including antisense oligonucleotides and ribozyme- or antibody-encoding sequences, can be administered in a range of about 100 ng to about 200 mg of DNA for local administration. Suitable concentrations range from about 500 ng to about 50 mg, about 1 μ g to about 2 mg, about 5 μ g to about 500 μ g, and about 20 μ g to about 100 μ g of DNA. Factors such as method of action and efficacy of transformation and expression are considerations which will affect the dosage required for ultimate efficacy of the huBUB1 composition. If greater expression is desired over a larger area of tissue, larger amounts of a huBUB1 composition or the same amount administered successively, or several administrations to different adjacent or close tissue portions of, for example, a tumor site, may be required to effect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect.

Expression of an endogenous huBUB1 gene in a cell can be altered by introducing in frame with the endogenous huBUB1 gene a DNA construct comprising a huBUB1 targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site by homologous recombination, such that a homologously recombinant cell comprising a new huBUB1 transcription unit is formed. The new tran-

scription unit can be used to turn the huBUB1 gene on or off as desired. This method of affecting endogenous gene expression is taught in U.S. Pat. No. 5,641,670, which is incorporated herein by reference.

The targeting sequence is a segment of at least 10, 12, 15, 20, or 50 contiguous nucleotides selected from the nucleotide sequence shown in SEQ ID NO:1. The transcription unit is located upstream of a coding sequence of the endogenous huBUB1 gene. The exogenous regulatory sequence directs transcription of the coding sequence of the huBUB1 gene.

The present invention also provides methods of screening test compounds for the ability to decrease or inhibit huBUB1 kinase activity or to interfere with huBUB1-huBUB3 binding. The test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art.

huBUB1 protein is a target for huBUB1 kinase activity in an autophosphorylation reaction. huBUB1-dependent phosphorylation is not be limited to huBUB1 protein, but can be successfully directed towards exogenous substrates. Thus, huBUB1 kinase activity can be employed in the development of screening assays directed at the identification of biochemical inhibitors of huBUB1. huBUB1 kinase inhibitors can be used to develop novel therapeutic approaches to cancer and other hyperproliferative disorders, such as psoriasis. Screening assays can employ other kinase substrates, such as truncated huBUB1 polypeptides, fusion proteins, synthetic peptides, or substrates unrelated to huBUB1. These alternative huBUB1 kinase substrates may have specific attributes (cost, ease of use, relative efficiency) which may make them preferable to the use of huBUB1 autophosphorylation as a means of monitoring kinase activity. Alternatively, knowledge of activity of a given protein as a huBUB1 kinase substrate has applications in the discovery of novel targets for diagnostic and/or therapeutic applications related to huBUB1. For example, a high-throughput filter-based library screening approach, which employs biochemical activity of specific known kinases to identify bacterial and/or phage clones expressing cloned substrates of kinase, can be used to identify biologically relevant substrates of huBUB1 kinase. These cloned substrates can be characterized and employed in the further development of screening assays and/or pharmaceutical targets.

For example, compounds which decrease the kinase activity of huBUB1 or of a huBUB1-huBUB3 complex can be identified by contacting huBUB1 or a huBUB1-huBUB3 complex with a test compound and determining the kinase activity of the huBUB1 or huBUB1-huBUB3 complex. Any in vitro kinase assay known in the art, such as taught in W096/36642, can be used for this purpose (see also Example 7). Phosphorylation of a substrate, such as huBUB1 itself or a synthetic peptide substrate based on huBUB1 sequences shown in SEQ ID NO:2, or a kinase substrate such as PHAS-1, can be measured. Optionally, the substrate can comprise a detectable label, such as biotin, for use in a purification or separation step. A test compound which decreases kinase activity of huBUB1 or of the huBUB1-huBUB3 complex is identified as a candidate therapeutic agent.

Test compounds can also be screened for the ability to interfere with huBUB1-huBUB3 binding, in order to

develop pharmaceuticals directed at inhibiting huBUB1 and/or huBUB3 function in cells. Such inhibitors can be, for example, polypeptides, small peptides, peptoids, or other peptide analogs or other chemical inhibitors. Some of these inhibitors, such as related peptides or fusion proteins, can be developed rationally on the basis of knowledge of the sequences of huBUB1 and huBUB3 which are disclosed herein. Alternatively, a random array of compounds can be screened for the ability to compete in a huBUB1-huBUB3 binding assay.

A test compound can be contacted with a mixture of huBUB3 protein and a contiguous sequence selected from the huBUB1 amino acid sequence shown in SEQ ID NO:2. These molecules can be produced recombinantly or can be synthesized using standard chemical methods. The proteins can be prebound prior to the step of contacting the test compound. Alternatively, the test compound can contact one of the proteins before the second protein is added.

The proteins can be in solution or one protein can be bound to a solid support. The proteins can be unlabeled or labeled, for example, with a radioactive, fluorescent, or other detectable marker. They can be fusion proteins comprising huBUB1 or huBUB3 fused to another protein with or without a detectable enzymatic activity.

In one embodiment, the amount of at least one of the two proteins that is bound or unbound in the presence of the test compound is then measured. A number of methods can be used to measure the amount of proteins or dimers. For example, the relative concentration of proteins bound to unbound can be detected by examining the apparent molecular masses of the molecules by size exclusion chromatography or by polyacrylamide gel electrophoresis under non-reducing conditions. Other methods of measuring binding or dissociation of the proteins will readily occur to those of ordinary skill in the art and can be used. A test compound which diminishes the quantity of the first protein bound to the second protein, or which displaces the first protein bound to the second protein, or which prevents the first protein from binding to the second protein is identified as a candidate therapeutic agent.

According to the present invention a method is also provided of using the yeast two-hybrid technique to screen for test compounds which interfere with huBUB1-huBUB3 binding. The yeast two-hybrid technique is generically taught in Fields, S. and Song, O., *Nature* 340, 245-46, 1989.

In a preferred embodiment, a cell is contacted with a test compound. The cell comprises two fusion proteins, which can be supplied to the cell by means of recombinant DNA constructs. The first fusion protein comprises a DNA-binding domain. The second fusion protein comprises a transcriptional activating domain. The first fusion protein also comprises either (i) at least a portion of huBUB1 that binds to huBUB3 or (ii) at least a portion of huBUB3 that binds to huBUB1. If the first fusion protein comprises at least the portion of huBUB1 that binds to huBUB3, then the second fusion protein comprises at least the portion of huBUB3 that binds to huBUB1. If the first fusion protein comprises at least the portion of huBUB3 that binds to huBUB1, then the second fusion protein comprises at least the portion of huBUB1 that binds to huBUB3. The cell also comprises a reporter gene comprising a DNA sequence downstream from a DNA element to which the DNA binding domain of the first fusion protein binds.

When the huBUB3 and huBUB1 regions are bound together, the DNA binding domain and the transcriptional activating domain will be in close enough proximity to

reconstitute a transcriptional activator capable of initiating transcription of a detectable reporter gene in the cell. The expression of the reporter gene in the presence of the test compound is then measured. A test compound that increases the expression of the reporter gene is a potential drug for increasing huBUB1-huBUB3 binding. A test compound that decreases the expression of the reporter gene is a potential drug for decreasing huBUB1-huBUB3 binding.

Many DNA binding domains and transcriptional activating domains can be used in this system, including the DNA binding domains of GALA, LexA, and the human estrogen receptor paired with the acidic transcriptional activating domains of GAL4 or the herpes virus simplex protein VP16 (see, e.g., G. J. Hannon et al., *Genes Dev.* 7, 2378, 1993; A. S. Zervos et al., *Cell* 72, 223, 1993; A. B. Votjet et al., *Cell* 74, 205, 1993; J. W. Harper et al., *Cell* 75, 805, 1993; B. Le Douarin et al., *Nucl. Acids Res.* 23, 876, 1995). A number of plasmids known in the art can be constructed to contain the coding sequences for the fusion proteins using standard laboratory techniques for manipulating DNA (see, e.g., Example 1, below).

Suitable detectable reporter genes include the *E. coli* lacZ gene, whose expression can be measured calorimetrically (see, e.g., Fields and Song, supra), and yeast selectable genes such as HIS3 (Harper et al., supra; Votjet et al., supra; Hannon et al., supra) or URA3 (Le Douarin et al., supra). Methods for transforming cells are also well known in the art. See, e.g., A. Hinnen et al., *Proc. Natl. Acad. Sci. U.S.A.* 75, 1929-1933, 1978. The test compound can comprise part of the cell culture medium or it may be added separately.

The invention also provides methods of increasing the sensitivity of a tumor to a metabolic inhibitor. Normal cell division includes a highly controlled segregation of subcellular components, especially chromosomes and spindle pole bodies, a process which requires the function of microtubules. In normal cells, the presence of microtubule poisons arrests cell division prior to segregation of these components. In this manner, cells refrain from attempting to segregate these components under conditions which might affect the normal fidelity of this segregation.

In mutant cells lacking huBUB1 (and/or other genes known to function in this pathway such as huBUB3), a signal transduction pathway which senses proper microtubule function is absent. Thus, mutant cells treated with these drugs fail to regulate cell cycle progression. In this case, cell division occurs without proper segregation of subcellular components, and progeny cells may inherit a random fraction of genetic material (ranging from none to all), and may inherit one, none or both spindle poles. If progeny cells retain a less than complete complement of chromosomes and none or two spindle pole bodies, resulting cells are fated to die, either through loss of essential genes, through lack of spindle pole bodies, or through the catastrophic effects of a subsequent multipolar mitosis. This phenomenon is termed "mitotic catastrophe."

Mitotic catastrophe can be exploited to enhance the cytotoxic effect of anti-tumor agents on cancer cells to known microtubule poisons. Specifically, mutations in huBUB1 and functionally related genes (e.g., huBUB3) can determine the relative sensitivity of cells to microtubule poisons. In humans, the mutant status of huBUB1 and/or other genes can determine the relative cytotoxic effect of microtubule poison treatment in cancer chemotherapy. Such an effect may account for the difference between partial response and a complete remission in microtubule poison-mediated cancer chemotherapy. At the present time, the

precise mechanism of tumor cytotoxicity by microtubule poisons in cancer chemotherapy is relatively poorly understood. Inactivation of huBUB1 and/or other genes can be used to increase the relative sensitivity of many tumors to microtubule poisons, such as vinblastin, taxol, vincristine, and taxotere. Treatment of tumors comprising huBUB1 mutant cells with these agents can induce gross failure of mitotic segregation of subcellular components, thereby producing profound cytotoxicity. In contrast, treatment of non-mutant cells can induce transient cell cycle delay, from which cells can immediately recover following termination of treatment. Thus, the mutational status of huBUB1 can be determined to indicate which chemotherapeutic regimes should be used. For example, since wild-type huBUB1 confers resistance to microtubule poisons, the finding of a mutation in huBUB1 in a tumor indicates that such agents could be employed effectively to treat the tumor. In contrast, finding a wild-type huBUB1 will suggest use of other agents.

The invention also provides a novel chemotherapeutic regimen for treating neoplasia or its symptoms, in which tumor cells with a wild-type copy of the huBUB1 gene can be induced to undergo a lethal mitotic catastrophe effect in the presence of microtubule inhibitors. This can be accomplished by administering one or more biochemical inhibitors of huBUB1 and/or huBUB3 function, as well as one or more microtubule poisons. Inhibitors of huBUB1 and/or huBUB3 generate a transient loss of huBUB1 function analogous to that seen in genetically huBUB1-mutant cells, thereby generating a failure to properly regulate cell cycle when confronted with a microtubule poison. The resulting cytotoxicity resulting from failure of mitotic segregation would parallel that seen in huBUB1 mutant cells, with the added benefit that upon removal of the huBUB1/huBUB3 inhibitor, cells would return to a genetically stable state. In this manner, a transient inhibition of this pathway can be used to exploit the normal requirement of loss of huBUB1 function for the chemotherapeutic efficacy of microtubule poisons.

huBUB1 or huBUB3 inhibitors can be identified, for example, by kinase screening assays or by interference with huBUB1-huBUB3 binding, as described herein. Inhibitors can be added together, separately, or sequentially with the microtubule poison(s), as is desired. It is expected that the class of compounds including huBUB1/huBUB3 biochemical inhibitors described here would be used as adjuvants to normal cancer chemotherapy. Treated cells would therefore not be expected to express the constitutive genetic instability commonly observed in cancer cells. Cells transiently treated with huBUB1/huBUB3 inhibitors would be expected to return to a genetically stable state following cessation of treatment.

According to another aspect of the invention, potential drugs can be screened for utility as anti-cancer agents by the ability to suppress the expression or function of huBUB1 protein. Thus potential drugs can be contacted with cells and the expression of huBUB mRNA or protein monitored. This can be accomplished by well known techniques in the art, such as Northern blots, immunoprecipitation, immunoblots, etc. Any technique which utilizes a human huBUB1 nucleic acid probe or an antibody specific for human huBUB1 protein can be used. Other techniques, such as quantitative RT PCR can also be employed. In addition, in vitro techniques can be employed for testing the ability of candidate drugs to inhibit huBUB1 kinase activity or binding to huBUB3. Such assays are well within the skill of the art, once provided with the full sequence of the huBUB1 gene and protein. In addition, a yeast two-hybrid system can be

used wherein one of the partners comprises all or a portion of huBUB1 and one of the partners comprises all or a portion of huBUB3. A cell which contains both of these partners can be contacted with test compounds and the loss or diminution of transactivation of the reporter gene can be monitored.

A huBUB1 subgenomic polynucleotide can also be delivered to subjects for the purpose of screening test compounds for those which are useful for enhancing transfer of huBUB1 subgenomic polynucleotides to the cell or for enhancing subsequent biological effects of huBUB1 subgenomic polynucleotides within the cell. Such biological effects include hybridization to complementary huBUB1 mRNA and inhibition of its translation, expression of a huBUB1 subgenomic polynucleotide to form huBUB1 mRNA and/or huBUB1 protein, and replication and integration of a huBUB1 subgenomic polynucleotide. The subject can be a cell culture or an animal, preferably a mammal, more preferably a human.

Test compounds which can be screened include any substances, whether natural products or synthetic, which can be administered to the subject. Libraries or mixtures of compounds can be tested. The compounds or substances can be those for which a pharmaceutical effect is previously known or unknown. The compounds or substances can be delivered before, after, or concomitantly with a huBUB1 subgenomic polynucleotide. They can be administered separately or in admixture with a huBUB1 subgenomic polynucleotide.

Integration of a delivered huBUB1 subgenomic polynucleotide can be monitored by any means known in the art. For example, Southern blotting of the delivered huBUB1 subgenomic polynucleotide can be performed. A change in the size of the fragments of a delivered polynucleotide indicates integration. Replication of a delivered polynucleotide can be monitored inter alia by detecting incorporation of labeled nucleotides combined with hybridization to a huBUB1 probe. Expression of a huBUB1 subgenomic polynucleotide can be monitored by detecting production of huBUB1 mRNA which hybridizes to the delivered polynucleotide or by detecting huBUB1 protein. huBUB1 protein can be detected immunologically. Thus, the delivery of huBUB1 subgenomic polynucleotides according to the present invention provides an excellent system for screening test compounds for their ability to enhance transfer of huBUB1 subgenomic polynucleotides to a cell, by enhancing delivery, integration, hybridization, expression, replication or integration in a cell in vitro or in an animal, preferably a mammal, more preferably a human.

The following examples are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

EXAMPLE 1

This Example demonstrates isolation of a human cDNA sequence encoding huBUB1.

A human cDNA sequence of huBUB1 was determined (see SEQ ID NO:1). The complete mRNA corresponding to this sequence was examined by Northern Blotting and found to be of ~3.5 kb in length. This mRNA is of sufficient length to encode a protein of ~1000 amino acids. The predicted translation product of the isolated cDNA includes a protein kinase domain. This human gene sequence is unique and no match has been identified to date using homology searches of human sequences in public databases.

EXAMPLE 2

This example demonstrates that huBUB1 is present in a complex with huBUB3.

Four plasmids based on the commercial vector pCR3.1 (Invitrogen) were constructed. These plasmids encode wild-type huBUB1 (p385-1), huBUB1 carrying a dinucleotide substitution in the kinase motif resulting in a lysine to alanine substitution of amino acid 821 (p403-1; see FIG. 1), wild type huBUB3 (p291-2), and an epitope tagged FLAG-huBUB3 variant protein (p221-2), which encodes three copies of the FLAG epitope followed by a glycine linker region fused in the proper reading frame to the 5' terminus of the huBUB3 ORF.

The region containing the glycine-rich ATP binding loop and active site lysine are depicted in FIG. 1. Conserved amino acid residues (*) and similar residues (.) are indicated. There are five candidate active site lysine residues in this region of the huBUB1 gene. Based on a comparison with murine and *S. cerevisiae* genes and on local amino acid context of these huBUB1 lysine residues, lysine 821 in the huBUB1 kinase motif (... VLKQV ...) (SEQ ID NO:7) was targeted for site-directed mutagenesis using an overlapping PCR strategy. The AAA codon encoding lysine 821 was replaced by overlapping PCR with an AGC codon specifying alanine in the place of lysine. A mutant DNA fragment was then used to replace the corresponding fragment in p385-1. The resulting plasmid is referred to as p403-1.

The protein products of the genes encoded on these plasmids were then produced in vitro, using a commercial coupled transcription/translation kit (T7 TnT kit, Promega), either in the presence of ³⁵S-labeled methionine (Amersham), or in reactions without radioactive label. Reaction mixtures of 15 µl included 150 ng of huBUB1 wild-type or mutant plasmid and 60 ng of huBUB3 or FLAG-huBUB3 plasmid. Other components were added as recommended by the manufacturer. Coupled transcription/translation reactions were allowed to proceed for two hours at 30° C. before addition of TNES buffer (50 mM Tris 7.5, 100 mM NaCl, 2 mM EDTA, 1% NP-40) to halt in vitro protein synthesis reactions. All buffers were supplemented with 1 mM DTT.

Aliquots of translation mixes were immunoprecipitated using anti-FLAG monoclonal antibody coupled to agarose beads (Kodak). Translation mixes were clarified by centrifugation and the soluble fraction was incubated 1 hour at room temperature with 9 µl immunobeads previously washed in binding buffer (TNES), in a final volume of 100 µl. Beads were collected by brief centrifugation, and were washed sequentially to remove unbound material. Beads were washed twice in binding buffer (TNES), twice in TNES lacking NP40 (TNE) supplemented with 2 M NaCl, and twice in TNE buffer lacking EDTA and supplemented with 10 mM MgCl₂.

Beads were collected by brief centrifugation. Beads were then resuspended in 10 µl SDS-PAGE sample buffer and heated briefly to 95° C. to release associated proteins. Eluates were loaded on 10–20% SDS-PAGE gels (Novex). Following electrophoresis for 1 hour at 200V, gels were fixed in Coomassie destain solution and dried onto Whatman 3 mm chromatography paper prior to autoradiographic analysis.

An aliquot of ³⁵S-labeled reaction mixture was included on gels to indicate the relative efficiency of synthesis of the various plasmid products. Similar levels of labeled proteins were produced by the various plasmids used in these experiments. Following immunoprecipitation (IP) with anti-FLAG antibody beads, both FLAG-huBUB3 and huBUB1 material

were retained by the beads. In IP pellets lacking FLAG-huBUB3, relatively small amounts of huBUB1 were consistently observed.

These results indicate association between FLAG-huBUB3 and huBUB1. Because weakly associated proteins would have been removed by the 2M NaCl washes, the retention of both huBUB1 and FLAG-BUB3 by anti-FLAG agarose beads likely indicates a high affinity interaction between these components. Low levels of huBUB1 precipitation in the absence of FLAG-BUB3 likely represents low level protein aggregation occurring late in the assay, as similar levels could be detected in mock immunoprecipitation reactions lacking anti-FLAG immunobeads. ³⁵S-labeled huBUB1 lysine-821 to alanine mutant protein was also efficiently co-immunoprecipitated by FLAG-huBUB3, indicating that this amino acid is dispensable for huBUB1 interaction with FLAG-BUB3.

EXAMPLE 3

This example demonstrates autophosphorylation of huBUB1 and that lysine-821 is required for huBUB1 kinase activity.

Unlabeled in vitro transcription/translation reactions were prepared in parallel, immunoprecipitated, and further incubated with ³³P-ATP kinase substrate. Washed beads from unlabeled transcription/translation reactions were collected and further incubated in final wash buffer (TNE buffer lacking EDTA and supplemented with 10 mM MgCl₂) supplemented with 10 nM ³³P-labeled ATP (1 µCi per reaction), in a final volume of 25 µl. After 0.5 hr at room temperature, reactions were halted by addition of an excess of TNE buffer.

When wild-type huBUB1 was co-translated with FLAG-huBUB3, a ³³P-labeled band with a relative migration in SDS-PAGE gels identical to that of huBUB1 was detected, suggesting that wild-type huBUB1 is a kinase substrate. Omission of FLAG-huBUB3 resulted in failure to detect significant ³³P-labeled material. Because huBUB1 is poorly immuno-precipitated in the absence of FLAG-BUB3, this indicates that the labeled band represents huBUB1 translation product. Similarly, the absence of a huBUB1 band in reactions containing the huBUB1 lysine-821 to alanine mutant indicates failure of phosphorylation in this reaction.

This result indicates that the observed phosphorylation activity requires lysine-821 in the huBUB1 kinase motif. This result eliminates the possibility that the observed huBUB1 phosphorylation activity is due to a contaminating kinase co-immunoprecipitating with a FLAG-huBUB3/huBUB1 complex.

EXAMPLE 4

This example demonstrates the requirements for huBUB1 autophosphorylation activity.

Various experiments have been used to define the requirements of this autophosphorylation activity, including varying NaCl, MgCl₂, ATP, KCl, pH and time. The huBUB1 kinase is capable of autophosphorylation under a variety of conditions. Experiments have been conducted using an alternative immunoprecipitation protocol, in which an HA-epitope tag was introduced to the N-terminus of huBUB1, and complexes were precipitated using anti-HA monoclonal antibody and Protein G agarose beads (MB). HA-huBUB1 protein is also capable of autophosphorylation. Co-translation of huBUB3 may stimulate this autophosphorylation activity. This result may represent a biochemical

activation of the kinase by huBUB3 association, or improved efficiency of huBUB1 folding by huBUB3 co-translation. Because similar results were obtained independently of the epitope tagging strategy, we conclude the affinity of huBUB1 for huBUB3 and the observed autophosphorylation activity of huBUB1 are not due to unrelated artefacts.

EXAMPLE 5

This example demonstrates the identification of potential huBUB1 auto-phosphorylation sites.

Small amounts of unlabeled in vitro synthesized huBUB1/FLAG-BUB3 material were produced in scaled-up reactions and purified by immunoprecipitation. huBUB1 was auto-phosphorylated in the presence of ATP. Proteins were eluted from beads by heating in SDS-PAGE sample buffer containing DTT, and separated on 10–20% SDS-PAGE gels (Novex). Gels were stained with Coomassie blue.

Faint huBUB1 and FLAG-huBUB3 bands could be identified by relative migration, and the band corresponding to huBUB1 was excised from the gel. Protein in this gel slice was degraded using trypsin and the resulting peptide fragments were analyzed by electrospray mass spectrophotometry.

Species with a fragmentation pattern consistent with the presence of leucine and/or isoleucine could be detected, some of which could be matched to the expected masses of huBUB1 tryptic fragments as predicted from huBUB1 DNA sequence data. Of these peptides, a subset with a fragmentation pattern suggesting the presence of phosphate could be identified, including a peptide corresponding to the predicted mass of the huBUB1 peptide RVTISK (amino acids SEQ ID NO:8 210–216 of SEQ ID NO:2). This peptide is tentatively identified as a site of huBUB1 autophosphorylation. huBUB1 protein sequence resembles known mixed function kinases, which may phosphorylate serine, threonine or tyrosine. The actual phosphorylated residue may therefore represent modification of either threonine 213 or serine 215 in the huBUB1 sequence, or may represent modification of both of these residues.

EXAMPLE 6

This example demonstrates a method of screening for inhibitors of huBUB1 kinase activity.

A set of known compounds was screened using an in vitro huBUB1 kinase assay. Tested compounds included olomucine, myrecetin, hypericin, iodotubercidin, ellagic acid, emodin, and staurosporine. The relative specificity of these compounds towards known kinases has been reported in the literature. Some of these compounds can be classified as relatively specific kinase inhibitors (e.g., olomucine, myrecetin, ellagic acid), while other inhibitors are known to inhibit a broad range of kinase (e.g., hypericin, iodotubercidin, staurosporine).

Unlabeled FLAG-BUB3/BUB1 in vitro translation products were immunopurified with anti-FLAG. ³²P-ATP kinase reactions were performed in the presence and absence of inhibitors in 20 μ l reaction volumes. Labeled reaction products were separated on SDS-PAGE gels, and relative huBUB1 autophosphorylation was determined with the aid of a phosphorimager. An initial screen was conducted using 10 and 20 μ M of each compound. Compounds with a positive result in this screen were then retested at 5, 10 and 20 μ M (Table 1).

TABLE I

Dose	hypericin	iodotubercidin	staurosporine
5 μ M	0.63 \pm 0.07	0.56 \pm 0.08	0.45 \pm 0.11
10 μ M	0.47 \pm 0.13	0.41 \pm 0.09	0.49 \pm 0.11
20 μ M	0.36 \pm 0.05	0.32 \pm 0.05	0.26 \pm 0.04

huBUB1 kinase was clearly inhibited by hypericin, iodotubercidin, and staurosporine. These compounds fall in the class of compounds known as broad-range kinase inhibitors and are known inhibit a number of kinases at similar concentrations. These results demonstrate the successful use of screening assays directed towards the identification of biochemical inhibitors of the huBUB1 kinase.

EXAMPLE 7

This example demonstrates substrates of huBUB1 kinase.

Immunopurified FLAG-BUB3/BUB1 ³²P-ATP kinase reactions were assembled as described with the addition of 1 μ g of potential kinase substrate per 20 μ l kinase reaction. Parallel reactions in which huBUB1 was replaced with the previously described huBUB1-K821A mutant were also run. Reaction products were then separated on SDS-PAGE gels and visualized by autoradiography.

Tested protein targets included PHAS-I (Stratagene), casein, myelin basic protein (MBP), histone H1, and GST-p53 protein. Of these, only PHAS-I demonstrated clear huBUB1-dependent activity as a kinase substrate. Reactions containing the huBUB1-K821A mutant protein failed to produce labeled products, indicating that phosphorylation of PHAS-I requires huBUB1 activity.

These results demonstrate the utility of exogenous substrates in the determination of huBUB1 kinase activity. This result also demonstrates that a small scale screen can be directed at the identification of huBUB1 protein substrates, with potential applications towards the discovery of novel targets for novel diagnostic and therapeutic applications related to huBUB1.

PHAS-I substrate did not appear to interfere with the huBUB1 autophosphorylation reaction. This may indicate that the huBUB1 autophosphorylation reaction is more efficient than PHAS-I phosphorylation. This could occur if huBUB1 autophosphorylation occurs in a relatively efficient unimolecular reaction, through the phosphorylation of an individual monomer by the kinase domain of the same monomer. Alternatively, huBUB1 autophosphorylation could occur between separate huBUB1 monomers, in which case a freely associating exogenous substrate might be expected to more efficiently compete for huBUB1 phosphorylation.

EXAMPLE 8

This example demonstrates a small-scale screen to identify proteins and polypeptides which associate with or bind to huBUB1 and/or huBUB3. This screen can easily be adapted to identify small molecule inhibitors of huBUB1-huBUB3 binding, which could provide novel pharmaceuticals directed against huBUB1 and/or huBUB3 function. Using this assay, we define a subdomain of the N-terminus of huBUB1 as sufficient for huBUB3 association. This domain can serve as a useful source of peptide sequence with potential inhibitory properties in a huBUB1-huBUB3 interaction assay.

A number of huBUB1-encoding plasmids were produced. Plasmid p385-1, encoding full-length huBUB1 in the vector

pCR3.1 (Invitrogen), was employed as a backbone for further constructs. All constructs were in the proper orientation to be transcribed and translated in coupled reactions using the bacteriophage T7 promoter site located immediately upstream of the cloning site in vector pCR3.1. A full-length HA-epitope tagged huBUB1 plasmid was constructed (p396-1), which encodes a triple HA tag followed by a 6×glycine linker fused to the huBUB1 ATG start site. C-terminal truncation variants of this plasmid were also produced, including a construct encoding HA-BUB1 1-199 (p365-2), representing a deletion of the C terminus to an internal huBUB1 EcoRV site; HA-BUB1 1-400 (p377-2), representing a N-terminal MluNI huBUB1 fragment; and HA-BUB1 200-400 (p365-1), which fuses the HA tag to an internal EcoRV MluNI BUB1 fragment. These truncation variants include additional vector-encoded peptide sequences at their C-termini. Additional constructs used in the studies described here include pCR3.1-based human MAD2 and hu-rael expression plasmids (designated p344-6 and p375-1, respectively) produced by cloning PCR products derived from the open reading frames (ORFs) for these genes, as described in their respective Genbank entries (for human MAD2, HSRNAMAD and HSU65410; for hu-rael, HSU84720).

³⁵S-methionine-labeled proteins and polypeptides were then produced from these plasmids by coupled in vitro transcription/translation reactions (IVT), as described above. IVT reactions were immunoprecipitated (IP), using either anti-FLAG agarose beads (Kodak IBI) or anti-HA monoclonal antibody combined with Protein G beads (BMB). Beads were extensively washed as previously described to ensure the removal of non-specifically-associated proteins, including relatively stringent washes in 2 M NaCl buffer. Bound proteins were released from beads by heating samples in SDS-sample buffer and were analyzed on 10-20% SDS-PAGE gels. Aliquots of initial ³⁵S-labeled translation reactions were analyzed as well.

Polypeptides observed to associate with FLAG-BUB3 included full-length huBUB1, as well as huBUB1 truncation variants HA-BUB1 200-400 and HA-BUB1 1-400. Proteins not retained by FLAG-BUB3 included human MAD2, untagged huBUB3, and HA-BUB1 1-199.

A second set of experiments was used to demonstrate affinity of various polypeptides for HA-BUB1 1-400. Both huBUB3 and FLAG-BUB3 bound to HA-BUB1 1-400. Neither full-length huBUB1 or the product of the human rael gene were observed to associate with this domain of huBUB1.

The association of truncation variants of huBUB1 with FLAG-BUB3 under these relatively stringent conditions suggests that an N-terminal subdomain of huBUB1 encompassed by the HA-BUB1 200-400 truncation variant is sufficient for recognizing huBUB3. Similar results were obtained when the HA-BUB1 1-400 truncation variant was used independently to immunoprecipitate huBUB3 and FLAG-BUB3, demonstrating that these protein-protein interactions can be observed independently from the IP method.

Omission of the 2 M NaCl IP washes allowed the detection of some retention of HA-BUB1 1-199 by FLAG-

BUB3. The observation of relatively weak association of FLAG-BUB3 with HA-BUB1 1-199 vs. HA-BUB1 200-400 was somewhat surprising. Alignment of *S. cerevisiae* and huBUB1 sequences suggested that a domain in the first 200 amino acids of huBUB1 retains the most highly conserved segments of amino acid sequences between these proteins, defining a conserved domain that we initially presumed would be sufficient to mediate high affinity huBUB3 interaction. A relatively weakly conserved second huBUB1 homolog has been described (huBUB1R1; Cahill et al., *Nature* 392:300-303), and a second domain of additional amino acid sequence homology between huBUB1 and huBUB1R1 was identified near the N-terminus, corresponding to sequences retained in the HA-BUB1 200-400 construct. We conclude that a domain in this region of huBUB1 is sufficient for high affinity huBUB1-huBUB3 association, but that additional protein-protein contacts are likely made by a domain present in the HA-BUB1 1-199 construct.

While huBUB3 clearly associates with huBUB1, huBUB3 was excluded from huBUB1-FLAG-BUB3 anti-FLAGIP complexes. This result suggests that huBUB1 complexes retain a single huBUB3 monomer and that huBUB3 does not freely self-associate. When huBUB3 was present, the yield of huBUB1 in FLAG-BUB3 complexes was reduced. This result was reproducibly obtained and indicates competition between FLAG-BUB3 and huBUB3 in the translation mix for association with huBUB1.

HA-BUB1 1-400 fails to associate with full-length huBUB1 in the presence of BUB3. Other experiments using full-length HA-BUB1 indicated that untagged huBUB1 was similarly excluded from HA-BUB1/BUB3 anti-HA IP complexes. These results suggest in turn that huBUB3 complexes retain a single huBUB1 monomer, and that huBUB1 does not freely self-associate. Accordingly, we conclude that the huBUB1-huBUB3 complexes studied in these experiments consist of a single monomer of each protein.

The association of hu-rael translation product with the huBUB3 binding domain of huBUB1 was also investigated. Homology searches identified both the huBUB3 and hu-rael proteins as candidate ligands for huBUB1, based on the fact that both proteins express significant homology to *S. cerevisiae* BUB3. We have not identified conditions which allow significant association to be detected between the hu-rael protein and huBUB1. Both hu-rae-1 and huBUB3 consist almost exclusively of WD40 (trp-asp) repeat motifs. Hu-rae-1 is also structurally related to the *S. pombe* rael gene and the *S. cerevisiae* YET7 gene, which have not been reported to play roles in signaling kinetochore function.

We also studied the association of the human MAD2 gene product with huBUB1 and huBUB3. Murine BUB1 and human MAD2 have each been shown to associate with human kinetochores. huBUB1 and huBUB3 are localized to mitotic nuclei in overexpressing cells. Strong homology to *S. cerevisiae* MAD2 suggests that the human MAD2 homolog functions in the same regulatory pathway. We have not identified conditions where a clear complex of human MAD2 and huBUB1 or huBUB3 proteins can be observed. At this point, we cannot rule out that MAD2 associates with huBUB1 and/or huBUB3 under some other conditions, either directly or in the form of a tertiary complex with other unknown proteins.

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Ile	Gln	Glu	Lys	Ser	Pro	Lys	Gln	Ala	Leu	Ser	Ser	His	Met	Tyr	Ser
			660					665					670		
Ala	Ser	Leu	Leu	Arg	Leu	Ser	Gln	Pro	Ala	Ala	Gly	Gly	Val	Leu	Thr
			675				680					685			
Cys	Glu	Ala	Glu	Leu	Gly	Val	Glu	Ala	Cys	Arg	Leu	Thr	Asp	Thr	Asp
	690				695						700				
Ala	Ala	Ile	Ala	Glu	Asp	Pro	Pro	Asp	Ala	Ile	Ala	Gly	Leu	Gln	Ala
	705				710					715					720
Glu	Trp	Met	Gln	Met	Ser	Ser	Leu	Gly	Thr	Val	Asp	Ala	Pro	Asn	Phe
			725						730					735	
Ile	Val	Gly	Asn	Pro	Trp	Asp	Asp	Lys	Leu	Ile	Phe	Lys	Leu	Leu	Ser
			740					745					750		
Gly	Leu	Ser	Lys	Pro	Val	Ser	Ser	Tyr	Pro	Asn	Thr	Phe	Glu	Trp	Gln
		755						760				765			
Cys	Lys	Leu	Pro	Ala	Ile	Lys	Pro	Lys	Thr	Glu	Phe	Gln	Leu	Gly	Ser
		770				775					780				
Lys	Leu	Val	Tyr	Val	His	His	Leu	Leu	Gly	Glu	Gly	Ala	Phe	Ala	Gln
	785				790					795					800
Val	Tyr	Glu	Ala	Thr	Gln	Gly	Asp	Leu	Asn	Asp	Ala	Lys	Asn	Lys	Gln
			805						810					815	
Lys	Phe	Val	Leu	Lys	Val	Gln	Lys	Pro	Ala	Asn	Pro	Trp	Glu	Phe	Tyr
			820					825					830		
Ile	Gly	Thr	Gln	Leu	Met	Glu	Arg	Leu	Lys	Pro	Ser	Met	Gln	His	Met
		835					840					845			
Phe	Met	Lys	Phe	Tyr	Ser	Ala	His	Leu	Phe	Gln	Asn	Gly	Ser	Val	Leu
			850			855					860				
Val	Gly	Glu	Leu	Tyr	Ser	Tyr	Gly	Thr	Leu	Leu	Asn	Ala	Ile	Asn	Leu
			865			870				875					880
Tyr	Lys	Asn	Thr	Pro	Glu	Lys	Val	Met	Pro	Gln	Gly	Leu	Val	Ile	Ser
			885						890					895	
Phe	Ala														

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Ile Phe Thr Ala Lys Cys Glu Thr Ser Gly Phe Gln Cys Val Glu Met
 965 970 975

Leu Ser Asn Lys Pro Trp Asn Tyr Gln Ile Asp Tyr Phe Gly Val Ala
 980 985 990

Ala Thr Val Tyr Cys Met Leu Phe Gly Thr Tyr Met Lys Val Lys Asn
 995 1000 1005

Glu Gly Gly Glu Cys Lys Pro Glu Gly Leu Phe Arg Arg Leu Pro His
 1010 1015 1020

Leu Asp Met Trp Asn Glu Phe Phe His Val Met Leu Asn Ile Pro Asp
 1025 1030 1035 1040

Cys His His Leu Pro Ser Leu Asp Leu Leu Arg Gln Lys Leu Lys Lys
 1045 1050 1055

Val Phe Gln Gln His Tyr Thr Asn Lys Ile Arg Ala Leu Arg Asn Arg
 1060 1065 1070

Leu Ile Val Leu Leu Leu Glu Cys Lys Arg Ser Arg Lys
 1075 1080 1085

<210> SEQ ID NO 3
 <211> LENGTH: 2619
 <212> TYPE: DNA
 <213> ORGANISM: Homo sapien

<400> SEQUENCE: 3

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ctctccgtg aagttcagcc ccaacacctc ccagttctct cttgtctct cctgggacac 180

gtccgtgcgt ctctacgatg tgccggccaa ctccatgcgg ctcaagtacc agcacaccgg 240

cgccgtcctg gactgcgcct tctacgatcc aacgcatgcc tggagtggag gactagatca 300

tcaattgaaa atgcatgatt tgaacactga tcaagaaaat cttgttggga cccatgatgc 360

ccctatcaga tgtgttgaat actgtccaga agtgaatgtg atggtcactg gaagttggga 420

tcagacagtt aaactgtggg atcccagaac tccttgaat gctgggacct tctctcagcc 480

tgaaaaggta tataccctct cagtgtctgg agaccggctg attgtggga cagcaggccg 540

cagagtgttg gtgtgggact tacggaacat gggttacgtg cagcagcgca gggagtccag 600

cctgaaatac cagactcgct gcatacgagc gtttccaaac aagcagggtt atgtattaag 660

ctctattgaa ggcgagtggt cagttgagta tttggacca agccctgagg tacagaagaa 720

gaagtatgcc ttcaaatgct acagactaaa agaaaataat attgagcaga ttaccacagt 780

caatgccatt tcttttcaca atatccacaa tacatttgcc acagtggtt ctgatggctt 840

tgtaaatatt tgggatccat ttaacaaaaa gcgactgtgc caattccatc ggtacccac 900

gagcatcgca tcaactgcct tcagtaatga tgggactacg cttgcaatag cgtcatcata 960

tatgtatgaa atggatgaca cagaacatcc tgaagatggt atcttcattc gccaaagtgc 1020

agatgcagaa acaaaaccca agtcaccatg tacttgacaa gatttcattt acttaagtgc 1080

catgttgatg ataataaaac aattcgtact cccaatggt ggatttatta ctattaaaga 1140

aaccaggga aatattaatt ttaataatt aacaacctga aaataatgga aaagaggttt 1200

ttgaattttt ttttttaaat aaacaccttc ttaagtgcac gagatgggtt gatggtttgc 1260

tgcattaaag gtatttgggc aaacaaaatt ggagggcaag tgactgcagt tttgagaatc 1320

agttttgacc ttgatgattt tttgtttcca ctgtggaaat aaatgtttgt aaataagtgt 1380

aataaaaatc cctttgcatt ctttctggac cttaaatggt agaggaaaag gctcgtgagc 1440

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catttggttc ttttgctggt tatagttgct aattctaaag ctgcttcaga ctgcttcag 1500
aggagggttaa tctacaatta aacaatattt cctcttgccc gtccattatt ttctgaagca 1560
gatgggtcoat catttcctgg gctgttaaac aaagcgaggt taaggtaga ctcttgggaa 1620
tcagctagtt ttcaatctta ttagggtgca gaaggaaaac taataagaaa acctccta 1680
atcattttgt gactgtaaac aattatttat tagcaaacaa ttgatcccag aaggggcaaat 1740
tgtttgagtc agtaatgagc tgagaaaaga cagagcatat ctgtgtattt ggaaaaataa 1800
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atgaattttt ggaacatta atgagggtta catatttctc tgacatttat atagttctta 1920
tgtccatttc agttgaccag ccgctgggtg ttaaagttaa aaagaaaaaa attatagtga 1980
gaatgagatt catttcaatg taatgcacta aagcagaaca cgaacttagc ttggcctatt 2040
ctaggtagtt ccaaatagta tttttgtgt caaactttaa aatttatatt aatttgcaaa 2100
tgtatgtctc tgagtaggac ttggacctt cctgagattt attttatccg tgatgtattt 2160
tttttaattc ttttgataca gagaagggtc ttttttttt taagtatttc agtgaaaact 2220
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atcctgtgaa agtggtttct ctatggaaag ctttgtttgc ttctacaaa tacatgctta 2340
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tctgacgtat ttccccttct gtcccctagt aagcccagtt gctgtatctg aacagtttga 2520
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<210> SEQ ID NO 4

<211> LENGTH: 328

<212> TYPE: PRT

<213> ORGANISM: Homo sapien

<400> SEQUENCE: 4

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Met Thr Gly Ser Asn Glu Phe Lys Leu Asn Gln Pro Pro Glu Asp Gly
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Ile Ser Ser Val Lys Phe Ser Pro Asn Thr Ser Gln Phe Leu Leu Val
 20            25            30
Ser Ser Trp Asp Thr Ser Val Arg Leu Tyr Asp Val Pro Ala Asn Ser
 35            40            45
Met Arg Leu Lys Tyr Gln His Thr Gly Ala Val Leu Asp Cys Ala Phe
 50            55            60
Tyr Asp Pro Thr His Ala Trp Ser Gly Gly Leu Asp His Gln Leu Lys
 65            70            75            80
Met His Asp Leu Asn Thr Asp Gln Glu Asn Leu Val Gly Thr His Asp
 85            90            95
Ala Pro Ile Arg Cys Val Glu Tyr Cys Pro Glu Val Asn Val Met Val
100           105           110
Thr Gly Ser Trp Asp Gln Thr Val Lys Leu Trp Asp Pro Arg Thr Pro
115           120           125
Cys Asn Ala Gly Thr Phe Ser Gln Pro Glu Lys Val Tyr Thr Leu Ser
130           135           140
Val Ser Gly Asp Arg Leu Ile Val Gly Thr Ala Gly Arg Arg Val Leu
145           150           155           160

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Val	Trp	Asp	Leu	Arg	Asn	Met	Gly	Tyr	Val	Gln	Gln	Arg	Arg	Glu	Ser
			165						170					175	
Ser	Leu	Lys	Tyr	Gln	Thr	Arg	Cys	Ile	Arg	Ala	Phe	Pro	Asn	Lys	Gln
			180				185						190		
Gly	Tyr	Val	Leu	Ser	Ser	Ile	Glu	Gly	Arg	Val	Ala	Val	Glu	Tyr	Leu
		195					200					205			
Asp	Pro	Ser	Pro	Glu	Val	Gln	Lys	Lys	Lys	Tyr	Ala	Phe	Lys	Cys	His
	210					215					220				
Arg	Leu	Lys	Glu	Asn	Asn	Ile	Glu	Gln	Ile	Tyr	Pro	Val	Asn	Ala	Ile
	225				230					235					240
Ser	Phe	His	Asn	Ile	His	Asn	Thr	Phe	Ala	Thr	Gly	Gly	Ser	Asp	Gly
			245						250					255	
Phe	Val	Asn	Ile	Trp	Asp	Pro	Phe	Asn	Lys	Lys	Arg	Leu	Cys	Gln	Phe
			260					265					270		
His	Arg	Tyr	Pro	Thr	Ser	Ile	Ala	Ser	Leu	Ala	Phe	Ser	Asn	Asp	Gly
		275					280					285			
Thr	Thr	Leu	Ala	Ile	Ala	Ser	Ser	Tyr	Met	Tyr	Glu	Met	Asp	Asp	Thr
		290				295					300				
Glu	His	Pro	Glu	Asp	Gly	Ile	Phe	Ile	Arg	Gln	Val	Thr	Asp	Ala	Glu
	305				310					315					320
Thr	Lys	Pro	Lys	Ser	Pro	Cys	Thr								
					325										

<210> SEQ ID NO 5
 <211> LENGTH: 23
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer for PCR amplification of huBUB1

<400> SEQUENCE: 5

atcattcatg gagacattaa gcc

23

<210> SEQ ID NO 6
 <211> LENGTH: 24
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Primer for PCR amplification of huBUB1

<400> SEQUENCE: 6

tttcatgtaa gagccaaaga gcat

24

<210> SEQ ID NO 7
 <211> LENGTH: 5
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapien

<400> SEQUENCE: 7

Val Leu Lys Gln Val
1 5

<210> SEQ ID NO 8
 <211> LENGTH: 7
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapien

<400> SEQUENCE: 8

Arg Val Ile Thr Ile Ser Lys
1 5

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<210> SEQ ID NO 9
 <211> LENGTH: 44
 <212> TYPE: PRT
 <213> ORGANISM: *S. cerevisiae*

<400> SEQUENCE: 9

Asp Leu Tyr Cys Ile Arg Gly Glu Leu Gly Glu Gly Tyr Ala Thr
 1 5 10 15

Val Tyr Leu Ala Glu Ser Ser Gln Gly His Leu Arg Ala Leu Lys Val
 20 25 30

Glu Lys Pro Ala Ser Val Trp Glu Tyr Tyr Ile Met
 35 40

<210> SEQ ID NO 10
 <211> LENGTH: 49
 <212> TYPE: PRT
 <213> ORGANISM: *Mus musculus*

<400> SEQUENCE: 10

Leu Val Tyr Val Asn His Leu Leu Gly Glu Gly Ala Phe Ala Gln Val
 1 5 10 15

Phe Glu Ala Ile His Gly Asp Val Arg Asn Ala Lys Ser Glu Gln Lys
 20 25 30

Cys Ile Leu Lys Val Gln Arg Pro Ala Asn Ser Trp Glu Phe Tyr Ile
 35 40 45

Gly

<210> SEQ ID NO 11
 <211> LENGTH: 49
 <212> TYPE: PRT
 <213> ORGANISM: *Homo sapien*

<400> SEQUENCE: 11

Leu Val Tyr Val His His Leu Leu Gly Glu Gly Ala Phe Ala Gln Val
 1 5 10 15

Tyr Glu Ala Thr Gln Gly Asp Leu Asn Asp Ala Lys Asn Lys Gln Lys
 20 25 30

Phe Val Leu Lys Val Gln Lys Pro Ala Asn Pro Trp Glu Phe Tyr Ile
 35 40 45

Gly

<210> SEQ ID NO 12
 <211> LENGTH: 46
 <212> TYPE: PRT
 <213> ORGANISM: *Homo sapien*

<400> SEQUENCE: 12

Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys Gly Ser Phe Gly Lys
 1 5 10 15

Val Met Leu Ala Asp Arg Lys Gly Thr Glu Glu Leu Tyr Ala Ile Lys
 20 25 30

Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu
 35 40 45

<210> SEQ ID NO 13
 <211> LENGTH: 49
 <212> TYPE: PRT
 <213> ORGANISM: *Homo sapien*

<400> SEQUENCE: 13

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Glu Cys Phe Glu Leu Leu Arg Val Leu Gly Lys Gly Gly Tyr Gly Lys
 1 5 10 15
 Val Phe Gln Val Arg Lys Val Thr Gly Ala Asn Thr Gly Lys Ile Phe
 20 25 30
 Ala Met Lys Val Leu Lys Lys Ala Met Ile Val Arg Asn Ala Lys Asp
 35 40 45
 Thr

<210> SEQ ID NO 14
 <211> LENGTH: 46
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapien

<400> SEQUENCE: 14

Asp Gln Phe Glu Arg Ile Lys Thr Leu Gly Thr Gly Ser Phe Gly Arg
 1 5 10 15
 Val Met Leu Val Lys His Lys Glu Thr Gly Asn His Tyr Ala Met Lys
 20 25 30
 Ile Leu Asp Lys Gln Lys Val Val Lys Leu Lys Gln Ile Glu
 35 40 45

<210> SEQ ID NO 15
 <211> LENGTH: 44
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapien

<400> SEQUENCE: 15

Val Ser Tyr Thr Asp Thr Lys Val Ile Gly Asn Gly Ser Phe Gly Val
 1 5 10 15
 Val Tyr Gln Ala Lys Leu Cys Asp Ser Gly Glu Leu Val Ala Ile Lys
 20 25 30
 Lys Val Leu Gln Asp Lys Arg Phe Lys Asn Arg Glu
 35 40

What is claimed is:

1. An isolated and purified subgenomic polynucleotide consisting essentially of a nucleotide sequence shown in SEQ ID NO: 1.

2. A DNA expression construct comprising an isolated and purified subgenomic polynucleotide consisting essentially of a nucleotide sequence shown in SEQ ID NO:1.

3. A host cell comprising an isolated and purified subgenomic polynucleotide consisting essentially of a nucleotide sequence shown in SEQ ID NO:1.

4. A method of expressing a huBUB1 subgenomic polynucleotide in a cell in vitro, comprising the step of:

delivering a huBUB1 subgenomic polynucleotide having a nucleotide sequence shown in SEQ ID NO:1 to the cell, wherein said polynucleotide sequence contains a complete open reading frame, whereby the huBUB1 subgenomic polynucleotide is expressed.

5. An isolated and purified subgenomic polynucleotide encoding a mutant huBUB1 polypeptide, consisting essentially of the nucleotide sequence shown in SEQ ID No. 1 except said polynucleotide contains a mutation and encodes a mutant huBUB1 polypeptide containing a mutation of the amino acid lysine at position 821 of SEQ ID No. 2, and wherein the mutant huBUB1 polypeptide contains an alanine at amino acid position 821 of SEQ ID No. 2.

6. An isolated oligonucleotide probe consisting essentially of at least 10 nucleotides complementary to a huBUB1 nucleic acid sequence to detect a mutation of huBUB1 at amino acid position 821 of SEQ ID NO:2.

7. A polynucleotide which encodes a mutant huBUB1 protein comprising an amino acid sequence of SEQ ID NO:2 except the encoded mutant huBUB1 protein contains an alanine at position 821 of SEQ ID NO:2.

8. A host cell comprising a construct which comprises a promoter; and the polynucleotide of claim 7.

9. A construct comprising: a promoter; and

a polynucleotide segment encoding a mutant huBUB1 protein comprising an amino acid sequence of SEQ ID NO:2 except the encoded mutant huBUB1 protein contains an alanine at position 821 of SEQ ID NO:2 wherein the polynucleotide segment is located downstream from the promoter, wherein transcription of the polynucleotide segment initiates at the promoter.

* * * * *

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